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(54) **Title:** $\alpha_v\beta_3$ INTEGRIN AS A PREDICTOR OF ENDOMETRIOSIS

(57) **Abstract**

Methods of predicting endometriosis comprise obtaining a sample of endometrium selected from menstrual cycle day 20 to 24, identifying the endometrial sample as nulliparous, contacting the endometrium with a monoclonal antibody for β_3 integrin, assaying for β_3 integrin and correlating the absence of β_3 integrin with endometriosis, wherein the endometrium is identified as mild/minimum endometriosis. A method of using monoclonal antibodies to predict endometriosis is also within the scope of the invention. Methods for detecting receptivity of mammalian endometrium to embryo implantation comprising obtaining a sample of the endometrium, contacting the endometrium with a monoclonal antibody for β_3 and detecting β_3 in the endometrium. The invention also provides for methods of diagnosing infertility in a mammal and methods of detecting the window of embryo implantation in endometrium. Methods of *in vitro* fertilization, methods of preventing embryo implantation and a method of monitoring endometrial maturation are also within the scope of the present invention. The present invention is also directed to contraceptives. Diagnostic kits useful in the practice of the methods of the invention are also provided.

$\alpha_v\beta_3$ INTEGRIN AS A PREDICTOR OF ENDOMETRIOSIS

REFERENCE TO APPLICATIONS

This application is a continuation in part of application Serial No. 897,706, filed June 12, 1992.

5 ## REFERENCE TO GOVERNMENT GRANTS

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10 United States Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Over the past decade, investigators have come to recognize the importance of the extracellular matrix (ECM) in
15 directing the growth, differentiation and function of the overlying epithelium. Getzenberg et al., "The Tissue Matrix: Cell Dynamics and Hormone Action", *Endocrine Rev.* **1990**, *11*:399. The interaction between cell and extracellular matrix (or substratum) is mediated by several classes of cell
20 adhesion molecules, one of the most important being the integrins. Albelda et al., "Integrins and Other Cell Adhesion Molecules", *FESEB J.* **1990**, 4:2868. Buck et al., "Integrin, a Transmembrane Glycoprotein Complex Mediating Cell-Substratum Adhesion", *J. Cell Sci. Suppl.* **1987**, *8*:231.
25 This diverse family of glycoprotein receptors is expressed on the cell membrane as heterodimeric α and β integrin subunits and is involved in both cell-cell and cell-substratum adhesion. Specific recognition and binding of extracellular

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matrix (ECM) components such as fibronectin (FN), laminin
(LM) and collagen (Col) transmit information to the
cytoskeletal structure, an interaction which may have major
roles in promoting hormone responsiveness and genomic
5   activation. Burridge et al., "Focal Adhesions:
Transmembrane Junctions Between the Extracellular Matrix and
the Cytoskeleton", *Ann. Rev. Cell. Biol.* **1988**, 4:487 and
Getzenberg et al. *supra*.

        Although extensive information exists about
10 specific integrin proteins, for example, Hemler, M.E. "VLA
Proteins in the Integrin Family: Structures, Functions and
Their Role on Leukocytes", *Annu. Rev. Immunol.* **1990,** 8:365,
little is known concerning the distribution of these
receptors in the female reproductive tract. In the uterus,
15 the endometrium, composed of glandular epithelium and
associated mesenchyme (stroma), maintains complex temporal
and spatial functions in response to the cyclic hormonal
milieu. While Tabibzadeh reported that dynamic alterations
in integrin expression accompany the other histologic changes
20 that temporally mark the menstrual cycle, Tabibzadeh, S.,
"Patterns of Expression of Integrin Molecules in Human
Endometrium Throughout the Menstrual Cycle", *Hum. Reprod.*
1992, 7:876, the search for morphological or biochemical
markers for uterine receptivity has been unsuccessful to date
25 as reported by Rogers and Murphy, "Uterine Receptivity for
Implantation: Human Studies", in *Blastocyst Implantation*
1989, Yoshinaga, K. ed., Serono Symposia, p. 231. Once such
markers are identified, their role in endometrial phenomena
including embryo implantation, fertility, contraception and
30 endometrial maturation and receptivity can likely also be
identified. Thus, as some integrins appear to meet the
criteria for markers of receptivity there is a great need for
methods of detecting integrin cell adhesion molecules in
endometrium.
35   Asynchrony between uterine and embryonic
development has been suggested as a cause of pregnancy loss,
on the basis of a misalignment between endometrial and

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embryonic receptivity, see Pope, et al., "Uterine Asynchrony:
A cause of Embryonic Loss", *Biol. Reprod.* 1988, *39*:999.

The relationship between minimal endometriosis and
nulliparity and infertility remains controversial. While
5 many cogent arguments exist to support this association, many
clinicians today still do not believe that minimal or mild
forms of the disease are detrimental to a couple's fertility.
Several studies comparing women with normal endometrium to
women with mild or minimal endometriosis have demonstrated a
10 decrease in cycle fecundity, such as success rates in donor
programs, IVF, or GIFT, supported by animal models. One
reason for this lack of acceptance is the finding of many
studies that expectant management yields pregnancy rates as
high as most currently used treatments.
15   Of the many mechanisms suggested to explain the
decline in cycle fecundity of endometriosis patients, a
defect in uterine receptivity has received perhaps the least
attention. Fedele reported specific changes in the native
endometrium in women with severe endometriosis, Fedele, L.,
20 et al., "Structural and Ultrastructural Defects in
Preovulatory Endometrium of Normo-Ovulating Infertile Women
with Minimal or Mild Endometriosis", *Fertil. Steril.* **1990**,
53:989. Yovich suggested that severe endometriosis was
associated with a defect in implantation, based on IVF-ET
25 experience, see Yovich et al., "Hormonal Profiles and Embryo
Quality in Women with Severe Endometriosis Treated by *In
Vitro* Fertilization and Embryo Transfer", *Fertil. Steril.*
1988, *50*:308. There is data from studies in the rabbit to
suggest that implantation is adversely affected by surgically
30 induced endometriosis, and that the effect is mediated by the
peritoneal fluid, see Hahn et al., "Experimental Evidence for
Failure to Implant as a Mechanism of Infertility Associated
with Endometriosis", *Am. J. Obstet. Gynecol.* **1986**, *155*:1109.

Endometriosis is a disorder that affects an
35 estimated 2-5% of the general fertile female population, yet
its prevalence in infertile women approaches 30-50%, Peterson
et al., "Laparoscopy of the infertile Patient", *Obstet.*

Gynecol. **1970**, *36*:667. While many affected women may have no symptoms, others suffer from dysmenorrhea (painful, difficult menstruation), dyspareunia (pain during intercourse), menstrual disturbances and infertility. That mild or minimal

5 endometriosis is associated with infertility has been suggested by numerous studies, Hasson H.M., "Incidence of Endometriosis in Diagnostic Laparoscopy", *J. Reprod. Med.* **1976**, *16*:135 and has been recently reviewed, Bancroft, et al., "Minimal/Mild Endometriosis and Infertility. A Review",

10 *Br. J. Obstet. Gynaecol.* **1989**, *96*:454. The mechanism by which minimal endometriosis causes infertility remains uncertain. Adverse effects on folliculogenesis, ovulation, ovum transport, fertilization, sperm quality, embryos, luteal phase function, and an increase in spontaneous abortion rates

15 have all been postulated. These effects may be mediated by elaboration of peritoneal factors such as prostaglandins, cytokines, and growth factors; activation of peritoneal macrophages, or alterations in immune function such as decreased activity of natural killer cells. Kurzrock et al.,

20 "LIF: Not Just a Leukemia Inhibitory Factor", *Endo. Rev.* **1990,** *12*:208. A few authors have suggested that uterine receptivity to the embryo might be primarily affected by the presence of endometriosis, Muscato, et al., "Sperm Phagocytosis by Human Peritoneal Macrophages: A Possible

25 Cause of Infertility in Endometriosis", *Am. J. Obstet. Gynecol.* **1982**, *144*:503 and Yovich et al., *supra;* and limited data from surgically induced endometriosis in animal models support this hypothesis, Hahn, et al., *supra.* Further, structural abnormalities have been described in the

30 endometrium of women with endometriosis compared to normal fertile controls, Fedele, et al., *supra.*

Numerous approaches have been investigated to identify women with endometriosis using non-surgical means. Serum markers such as OC-125, (Barbieri, et al. "Evaluation

35 of a Serological Test for the Diagnosis of Endometriosis using a Monoclonal Antibody OC-125", SGI Annual Meeting **1985**; March:331P (abstract)) is increased in women with

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endometriosis, though the overlap with normal or other
disease states severely limits its utility as a diagnostic
test, Hornstein, et al., "Menstrual Cyclicity of CA-125 in
Patients with Endometriosis", *Fertil. Steril.* **1992**, *58*:279.
5   Further, given its graduated elevations with severity of
disease, OC-125 has little utility in cases of minimal or
mild endometriosis. Other modalities such as MRI or
ultrasound also have their highest sensitivity in the
presence of advanced endometriosis. To date, no method other
10 than laparoscopy has been demonstrated to provide proven
efficacy for the diagnosis of minimal or mild stages of
endometriosis.

       While uterine receptivity remains a poorly
understood phenomenon, the initial attachment of embryo to
15 maternal uterine lining is thought to be a critical step in
the implantation process. A window of implantation has been
defined by descriptive studies from the 1950s as well more
recent studies using advanced reproductive technologies,
Hertig et al., 1956 and Navot et al., 1992. The presence of
20 an integrin cell adhesion molecule that reliably appears on
the endometrial epithelial cells after day 19 of the normal
menstrual cycle, corresponds to this putative time of
implantation, Lessey, et al., "Integrin Adhesion Molecules in
the Human Endometrium", *J. Clin. Invest.* **1992**, *90*:188. The
25 expression of this integrin, the $\alpha_v\beta_3$ vitronectin receptor,
is delayed in women with maturational delay of the
endometrium, (where endometrial histology is delayed or
retarded because of inadequate hormone levels or decreased
response to existing levels of hormones) as well. As it has
30 been suggested by some that infertility due to endometriosis
may reflect a defect in uterine receptivity, it is of
interest to investigate the expression of this protein in the
endometria of women with infertility. The use of integrins
as diagnostic tools to investigate uterine receptivity proves
35 to be of significant value in identifying affected
individuals and assist in understanding the etiology of
infertility associated with mild forms endometriosis.

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SUMMARY OF THE INVENTION

The present invention is directed to methods of predicting endometriosis. Methods of using monoclonal antibodies to β_3 integrin are also within the scope of the
5 invention.

The methods of predicting endometriosis comprise obtaining a sample of endometrium selected from menstrual cycle day 20 to 24, identifying the endometrial sample as nulliparous, contacting the sample with a monoclonal antibody
10 specific for β_3 integrin, assaying for β_3 integrin in the sample, and correlating the absence of β_3 integrin with endometriosis, wherein the endometrium is identified as mild/minimal endometriosis. The present invention is also useful in predicting endometriosis in the general infertile
15 female population.

The methods of using monoclonal antibodies to β_3 integrin to predict endometriosis comprise obtaining a sample of endometrium selected from menstrual cycle day 20 to 24, contacting the sample with a monoclonal antibody specific for
20 β_3 integrin, assaying for β_3 in the sample, correlating the absence of β_3 with endometriosis, wherein the endometrium is identified as nulliparous or having mild/minimal endometriosis.

The present invention is directed to methods of
25 detecting receptivity of endometrium to embryo implantation by detecting the β_3 subunit of the α_v/β_3 integrin in endometrium with a monoclonal antibody.

Methods of diagnosing fertility and methods of monitoring endometrial maturation in a mammal are also
30 provided by monitoring the appearance of the β_3 subunit of integrin in endometrium from a plurality of stages of the endometrial cycle. This is preferably done with a monoclonal antibody.

The present invention also provides methods of
35 detecting the optimal window of embryo implantation in the endometrium by detecting the β_3 subunit of integrin in an endometrial sample, preferably with a monoclonal antibody.

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Further aspects of the invention include methods of preventing embryo implantation by contacting the β_3 subunit of integrin in the endometrium with neutralizing Fab antibody fragments to β_3. Methods of *in vitro* fertilization are also
5 embodiments of the invention. These comprise detecting the β_3 subunit of integrin in an endometrial sample, fertilizing an egg *in vitro*, and introducing the zygote into the uterus having endometrial tissue expressing the β_3 subunit.

Contraceptive and diagnostic kits are also
10 contemplated hereby.

These and other aspects of the invention will become more apparent from the following detailed description when taken in conjunction with the following figures.

BRIEF DESCRIPTION OF THE DRAWINGS
15   Figure 1 depicts immunoperoxidase staining of normal endometrium. The photomicrographs depict the pattern of distribution for six different integrins that do not appear to change throughout the menstrual cycle. Dark areas represent positive staining, light areas represent absence of
20 stain (absence of specific integrin subunit). Immunohistochemical staining of the collagen/laminin receptor subunits: α_2(A), α_3(B), α_6(C), and β_4(D) shows prominent staining of epithelium (←) and microvessels (←) without significant stromal staining (*) for α_2, α_3, and β_4. Note
25 basolateral staining α_6 and basal staining for β_4. Staining for fibronectin receptor subunits α_4(E) is not present in the proliferative stage, α_5(F) shows predominant mesenchyme staining (*) with decreased epithelial staining (←). The immunoreactions (areas of dark staining) were developed by
30 avidin-biotin-peroxidase complex using diaminobenzadine as a chromogen. For greater sensitivity, no counterstain was applied.
Magnification: 125X.

Figure 2 shows photomicrographs of the
35 immunohistochemical staining for the integrin subunit α_1 in proliferative vs. secretory endometrium. The staining in the

glandular epithelium (←) was largely absent in the
proliferative phase (A), and pronounced in all sections after
menstrual cycle day 14 (B; day 20 endometrium). The
microvasculature (←) staining was also pronounced, and did
5 not change throughout the menstrual cycle. The staining
noted in secretory endometrial glands was significantly
higher than that of background (C). Magnification: 125X.

Figure 3 exhibits immunostaining of α_v and β_3 (the
two pairing subunits of the vitronectin receptor integrin) in
10 proliferative phase vs. secretory phase endometrium. The
staining intensity of α_v in the proliferative phase (A) was
judged as "+" for the stromal cells (*) and "$\pm$" for glandular
α_v (←). Immunostaining for α_v in day 22 endometrium (B)
demonstrates a significant increase in glandular staining
15 (example of "++" staining intensity). Likewise, the staining
for β_3 was absent in proliferative epithelium (C; ←) and was
notably increased in this day 22 secretory endometrium (D).
Magnification: 125X.

Figure 4 shows relative intensity of staining for
20 the epithelial α_v and β_3 subunits in 35 endometrial samples
throughout the menstrual cycle. The pattern of expression
for α_v is shown in A, shows a gradual increase in staining
throughout the menstrual cycle. In contrast, the pattern for
β_3 in B, shows a more abrupt rise in this integrin subunit
25 around day 20 of the menstrual cycle. Samples were staged
according to the last menstrual cycle. Sections were
assigned a score of 0 (-; negative), 1 ($\pm$; weak), 2 (+;
moderate) or 3 (++; strong), by a blinded observer, and
confirmed by a second observer.
30 Figure 5 depicts staining intensity of epithelial β_3
in 12 infertility patients with delayed endometrial
maturation. Endometrium was collected from women undergoing
evaluation for infertility. The biopsies were separated into
two groups based on the correlation between histologic
35 criteria and the menstrual cycle dating based on the time of
ovulation and/or the subsequent menstrual period. Patients
with endometrial biopsies 3 or more days "out of phase" (OOP

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group) were compared with 25 endometrial biopsies that were "in phase" (Normal) and shown in A. Sections were assigned a score of 0 (-; negative), 1 ($\pm$; weak), 2 (+; moderate) or 3 (++; strong), based on the intensity of epithelial β_3

5   staining. Examples of immunohistochemical staining of an "out of phase" biopsy (B) and a normal "in phase" sample (C) is included to contrast the epithelial β_3 staining in each group. Magnification: 400X.

Figure 6 exhibits immunoblot analysis of

10   proliferative and secretory endometrium, stained for the β_3 subunit. (A) Immunoblot of platelet extract (lane 1) compared with 2 samples from the early and mid proliferative phase (lanes 2,3) and from the luteal phase (lane 4 and 5; days 23 and 26, respectively) demonstrates a band at

15   approximately 95 kD molecular weight, corresponding to β_3. Samples of endometrium were partially digested with collagenase and the glandular elements obtained (B) using a modification of the methods of Satyaswaroop et al., "Isolation and Culture of Human Endometrial Glands", *J. Clin.*

20   *Endocr. Metab.* **1979**, *48*:639. The glands appear as hollow structures free from surrounding stroma. Immunofluorescence of samples from lanes 3 and 4 (C and D, respectively) corresponds to the absence or presence of the 95 kD band in A. Magnification: 400X.

25   Figure 7 is a scattergram showing the immunohistochemical staining for the β_3 integrin subunit in endometrial samples form day 1 to 28 of the cycle. All samples had known endometrial dating, and none were out of phase, histologically.

30   Figure 8 displays immunostaining for the β_3 subunit of the $\alpha_v\beta_3$ vitronectin receptor. Endometrial epithelial cells (arrowheads) underwent alterations in β_3 expression throughout the menstrual cycle. Samples were obtained from the proliferative phase (A), early secretory phase (B; day

35   18), and from the mid secretory phase (C; day 22). An endometrial biopsy from a patient with mild endometriosis (D) had no epithelial staining for β_3, despite being judged

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"normal, in phase day 22-23" by histologic criteria. The
immunoreactions (areas of brown staining) were developed by
the avidin-biotin-peroxidase complex using diaminobenzadine
as a chromagen. Vascular elements (asterisks) stained
5   positive for the β_3 subunit in all samples. For better
contrast, the sections were counterstained with methyl green.
Green staining signifies the absence of β_3 expression. The
patterns of staining in A and B correspond to HSCOREs of 0
and in "C" to an HSCORE of 2.6. Magnification: 200X.

10   Figure 9 is a comparison of immunostaining results
for endometrial β_3 integrin in normal fertile controls
(Control; n = 20), infertile women with endometriosis and
prior parity (Parous; n = 28) and nulliparous infertile women
with endometriosis (Nulliparous; n = 76). The unpaired t
15   test compares the mean of two groups and determines the
likelihood of the observed difference occurring by change;
the chance is reported as a p value. Using the unpaired t
test, the overall HSCORE was significantly lower in the
latter group compared with either of the other two. HSCORE
20   was calculated as described.

           Figure 10 displays the distribution of the α_4 and β_3
integrin subunits during the normal menstrual cycle. Note
the co-expression of both subunits corresponds to the
putative window of implantation, postovulatory days 6-9.

25   **DESCRIPTION OF PREFERRED EMBODIMENTS**
           The present invention is directed to methods of
predicting endometriosis. Methods of using monoclonal
antibodies to β_3 integrin are also within the scope of the
invention.
30   The methods of predicting endometriosis comprise
obtaining a sample of endometrium selected from menstrual
cycle day 20 to 24, identifying the endometrial sample as
nulliparous, contacting the sample with a monoclonal antibody
specific for β_3 integrin, assaying for β_3 integrin in the
35   sample, and correlating the absence of β_3 integrin with
endometriosis, wherein the endometrium is identified as

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....ld/minimal endometriosis. The sample of endometrium is
preferably selected from menstrual cycle days 20 to 24, more
preferably menstrual cycle days 20 to 23, even more
preferably menstrual cycle day 22. Cycle days 20-24
5   correspond to post-ovulatory days 6-10. The present
invention is also useful in predicting endometriosis in the
general infertile female population.

The methods of using monoclonal antibodies to β_3
integrin to predict endometriosis comprise obtaining a sample
10 of endometrium, contacting the sample with a monoclonal
antibody specific for β_3 integrin, assaying for β_3 in the
sample, correlating the absence of β_3 with endometriosis,
wherein the endometrium is identified as nulliparous or
having mild/minimal endometriosis.

15   The present invention is directed to methods of
detecting receptivity of mammalian endometrium including
obtaining a sample of endometrium, contacting the sample with
a monoclonal antibody for the β_3 subunit of integrin and
detecting the β_3 subunit.

20   For purposes of the present invention, the β_3
subunit may be β_3 alone or β_3 in combination with another
integrin subunit, α_v for example.

As used herein integrin is defined as a diverse
class of glycoprotein receptors expressed on the cell
25 membrane. Integrins are cell adhesion molecules of the
immunoglobulin superfamily. Integrins are composed of
heterodimeric α and β subunits and are involved in cell -
cell and cell - extracellular matrix adhesion. The integrin
family is a broadly distributed group of receptors composed
30 of non-covalently associated α/β heterodimer pairs that
mediate leukocyte - leukocyte and leukocyte - endothelial
cell adhesion, as well as cellular interactions with
extracellular matrix components such as collagen, laminin,
fibrinogen and fibronectin, and cell-cell interaction in
35 organized tissues.

While integrins are found on virtually all cell
types (the exception being red blood cells), expression of

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integrin subunits varies from cell type to cell type. In
human uterine endometrium, as determined herein, glandular
epithelial cells express primarily α_2, α_3 and α_6 integrin
subunits, which are collagen laminin receptors. Stromal
5   cells express predominantly α_5, a fibronectin receptor. The
presence of α_1 on glandular epithelial cells is menstrual
cycle specific, found only during the secretory phase.
Expression of both subunits of the vitronectin receptor,
α_v/β_3, also undergoes menstrual cycle specific changes on
10 endometrial epithelial cells. The expression of α_v increases
throughout the menstrual cycle while the β_3 subunit appears
abruptly on menstrual cycle day 20 on luminal and glandular
epithelial cells. α_4 expression was also found to turn off
on day 23. Assaying for the expression of β_3 with monoclonal
15 antibody SSA6 revealed β_3 expression from day 20 onward into
the cycle. α_4 expression begins on day 14 and is no longer
expressed after day 23, as determined by assaying for α_4 with
monoclonal antibody B5G10, provided by Martin Hemler, Dana
Farber Cancer Center, Boston, MA. Further, in accordance
20 with the present invention, it has been discovered that α_4
and β_3 expression overlap between days 19-21, the window of
implantation.

As a result, as seen in Figure 10, integrins define
the window of implantation in the fertile, reproductive aged
25 female. These cell adhesion molecules are dynamically
regulated around the time of implantation and serve as
excellent marker proteins for this time of the menstrual
cycle. As shown in Figure 10, the co-expression of α_4 and β_3
(representing two different integrin species) coincide
30 precisely with the known window of implantation (post
ovulatory days 6-9, based on the work of Hertig et al., 1956
and Navot et al., 1992). In contrast to the β_3 integrin
which appears to be disrupted in numerous females with
infertility, the α_4 integrin does not. While the process of
35 implantation will likely involve many different proteins or
factors, such as the co-expression of two different integrins
as α_4 and β_3, the loss of a single entity such as β_3 appears

to be sufficient for the loss of fertility seen in these patients with endometriosis.

The present invention is directed to endometrium of the uterus of a mammal. The uterine wall is largely smooth
5   muscle or myometrium. The endometrium, a glandular layer of variable thickness extremely sensitive to the hormones estrogen/progesterone, lines the myometrium. The endometrium is composed of several functional layers. The layer nearest the myometrium is termed the basalis layer, and the layer
10 closer to the surface known as the functionalis. This tissue is made of epithelial cells, stromal (or mesenchymal) cells and endometrial leukocytes. The epithelial cells are either glandular (forming glands beneath the surface of the endometrium) or luminal (lining the surface of the
15 endometrium). These different types of epithelium serve different purposes and staining patterns for different marker proteins are not always the same between glandular and luminal. It is the luminal surface that would encounter the human embryo first and is thought to be involved in initial
20 attachment. The endometrium of premenopausal girls and post-menopausal women is atrophic due to the lack of the hormones, estrogen and progesterone. In the reproductive-aged woman, the endometrium undergoes cyclic developmental changes based on the ovarian cycle of hormone release. The first day of
25 menstruation is the first day of the cycle; menstruation is generally completed by day 5. The endometrial growth then resumes under the influence of estrogen and progresses through the day 14, proliferative phase, and on to about day 28. From day 14 to day 28 the endometrium also shows signs
30 of increased gland growth and secretion, secretory phase, due largely to the influence of progesterone. During the follicular phase, while follicles are growing in the ovary, and estrogen is the dominant hormone, the endometrium grows thicker. With ovulation (typically day 14 of a 28 day cycle)
35 the women is exposed to estrogen plus progesterone. This is called the secretory or luteal phase, and is noted for a stereotypic series of histologic changes that proceeds as the

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cycle continues. These histologic changes are used by
pathologists to date the endometrium, a process that remains
controversial despite its use for the past 40 years. There
have been no reliable immunohistochemical markers reported
5 that have proven utility in dating the endometrium.

Luteal phase dysfunction (LPD) is a term for
developmental delay of the endometrium. It is a known cause
of infertility, because of dyssynchrony between the
fertilized egg and the endometrium. If an embryo is ready to
10 attach but the endometrium is delayed, then pregnancy is not
likely to occur. The causes for LPD include inadequate
hormonal output by the ovary, and may implicate defective
signaling from higher centers such as inadequate gonadotropic
hormone output from the pituitary or hypothalamus. LPD is a
15 known cause of infertility and spontaneous abortion and can
be corrected with hormone augmentation.

Embryo implantation stages include: apposition -
when the epithelial cells of the embryo attach to the outer
(luminal) epithelial cells of the maternal endometrial
20 surface; adhesion; and invasion of trophoblast into the
underlying stroma where it established itself and begins to
grow. Contact with maternal blood vessels is made to gain
nutrients and oxygenated blood and to rid itself of waste
products during the invasion stage. The stage of development
25 that the embryo reaches at the time of implantation is the
blastocyst stage, which occurs at the same time as hatching.
There is evidence that hatching is required before
implantation occurs, perhaps because the embryo must have its
epithelial cells exposed (out of the zona pellucida shell) to
30 interact with the maternal cell layers. As set forth herein,
this interaction occurs via integrins.

For purposes of the invention, endometriosis is
defined as an ectopic occurrence of endometrial tissue,
frequently forming cysts containing altered red blood cells.
35 Stages of endometriosis have been established by the American
Fertility Society, AFS. American Fertility Society: Revised
American Fertility Society Classification of Endometriosis:

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1985.   Fertil Steril 1985; 43:351-352.   The AFS defines stage
I endometriosis as mild and stage II endometriosis as
minimal.

5   As set forth in the present invention, infertility
is defined as diminished or absent fertility such that a
female mammal has adequate anatomical structures and
equivocal function with possibility of pregnancy that may not
proceed to term.   Nulliparous includes, and is not limited
to, endometrium from a female mammal who has not borne
10   offspring.   Nulliparous includes females who have conceived
offspring and have not carried beyond 20 weeks gestation,
instead having spontaneous abortions.   Parous is defined in
accordance with the present invention as having borne young,
alive or dead, after a gestation of 20 weeks and a weight of
15   500 grams, for humans.   Appropriate lengths of gestation and
weight are applicable to other mammals such that they would
be comparable to those set forth for humans.

        For purposes of the current invention, mammals
include, but are not limited to the Order Rodentia, such as
20   mice; Order Logomorpha, such as rabbits; more particularly
the Order Carnivora, including Felines (cats) and Canines
(dogs); even more particularly the Order Artiodactyla,
Bovines (cows) and Suines (pigs); and the Order
Perissodactyla, including Equines (horses); and most
25   particularly the Order Primates, Ceboids and Simoids
(monkeys) and Anthropoids (humans and apes).   The mammals of
most preferred embodiments are humans.   Monoclonal antibodies
useful in the practice of the invention include any
monoclonal antibodies having an affinity to or binding to the
30   β_3 subunit of integrin.   An example of such a monoclonal
antibody is SSA6.   Monoclonal antibody SSA6 may be produced
as described by Bennett et al., *PNAS* **1983**, *80*:2417 and Brass
et al., *J. Biol. Chem.* **1985**, *260*:7876.

        Monoclonal antibodies which recognize β_3 combined
35   with another integrin subunit may also be used.   One such
monoclonal antibody is 23C6, which may be prepared according
to the method of Davies et al., *J. Cell Biol.* **1989**, *109*:1817.

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Immunostaining with monoclonal antibodies such as 23C6 (specific to the intact α_v/β_3 integrin, i.e. the vitronectin receptor) produces the identical pattern as SSA6 which only measures the β_3 subunit. This demonstrates that while α_v

5 specific antibodies measure all the α_v containing integrins, antibodies which recognize the intact α_v/β_3 integrin or the β_3 subunit can be used to study this integrin (the α_v/β_3 "vitronectin receptor").

Other monoclonal antibodies can be used. The
10 preparation of monoclonal antibodies is known to those in the art. Particularly, the method of Kohler and Milstein, *Nature* **1975**, *256*:495 may be used to produce monoclonal antibodies for use in the invention.

Methods of obtaining endometrial tissue samples for
15 analysis, include any surgical and nonsurgical technique known in the art. Surgical methods include, but are not limited to biopsy, dilation and curettage. Nonsurgical methods include, but are not limited to, uterine washings and uterine brushings with immunocytochemical evaluation.

20   Methods of detecting β_3 in the endometrium include all methods of identifying glycoproteins known in the art. These methods include, but are not limited to, immunohistochemistry techniques such as immunoblotting or Western blotting, immunoperoxidase staining, fluorescein
25 labeling, diaminobenzadine and biotinylation.

Generally, immunohistochemistry involves staining cryosectioned tissue samples. As used herein, endometrium samples may be cryosectioned to about $4-8\mu$ thick. Endometrium is contacted with primary antibody, such as SSA6,
30 followed by contact with secondary antibody, such as biotinylated goat anti-mouse antibody. Endometrium is then incubated in avidin-conjugated horseradish peroxidase macromolecular complex followed by chromagen incubation, such as diaminobenzadine. Fluorescein may then be added to
35 observe integrin distribution.

Immunoblotting involves the analysis of protein, here integrin, on sodium dodecylsulfate-polyacrylamide gel

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electrophoresis SDS-PAGE. The gel is run under nonreducing conditions and the samples are transferred to a nitrocellulose membrane for example. The membrane is incubated in media containing primary antibody, such as SSA6.

5   The filter is developed using a secondary antibody, such as alkaline phosphatase-conjugated goat anti-mouse antibody.

The methods of the present invention directed to predicting endometriosis comprise obtaining a sample of endometrium, identifying the sample of endometrium as

10   nulliparous, contacting the sample wit. a monoclonal antibody specific for β_3 integrin, assaying for β_3 integrin in the sample, and correlating the absence of β_3 integrin with endometriosis, wherein endometriosis is mild/minimal endometriosis.

15   Methods of using monoclonal antibodies to β_3 integrin to predict endometriosis are also within the scope of the invention and comprise obtaining a sample of endometrium, identifying the sample of endometrium as nulliparous, contacting the sample with a monoclonal antibody

20   specific for β_3 integrin, assaying for β_3 integrin in the sample, correlating the absence of β_3 integrin with endometriosis, wherein the endometrium is identified as mild/minimal endometriosis.

The methods of diagnosing infertility and for

25   detecting the window for embryo implantation in the endometrium of a mammal are also within the scope of the invention. As provided herein, the β_3 subunit of integrin appears at day 20 of the menstrual cycle. It is also provided herein that α_v/β_3 on endometrial epithelium binds

30   fibronectin, vitronectin and osteopontin. These molecules may provide a bridge between the α_v/β_3 integrin of the endometrium and the embryo. Further, patients with luteal phase dysfunction have delayed endometrial maturation, infertility and negative staining for β_3 on days 20 through

35   24. Thus, the optimal time for fertility may be determined by repetitively testing endometrial samples at a plurality of stages in the menstrual cycle. As such, screening for β_3

provides a method of diagnosing infertility and for detecting
the window of embryo implantation in the endometrium. The
window of implantation is that time when the endometrium of
the uterus is available for embryo implantation. This window

5 is preferably from day 19 to day 23, and more preferably day
20 of the human menstrual cycle, marked by the expression of
α_v/β_3 integrin.

Similar cycles are known for other mammals - it is
within the ordinary skill in the art to adopt the foregoing

10 methodology to such cycles.

The present invention is also directed to methods
of *in vitro* fertilization. Once the β_3 subunit of integrin
is detected in an animal selected for pregnancy, a
fertilizable egg (or eggs) from the same or different animal

15 could be replaced into the uterus to establish pregnancy.
The egg and appropriate sperm are combined to produce a
zygote *in vitro*. For purposes of the invention, *in vitro*
fertilization may take place in a petri dish, in a test tube
or the like. In addition, *in vitro* fertilization may also

20 refer to independently adding eggs and sperm to the fallopian
tubes such that the zygote is formed therein. In any event,
the zygote is introduced to the uterus of the animal selected
for pregnancy and monitored for implantation into the
endometrium of the uterine wall.

25   Alternatively, the invention is directed to methods
of preventing embryo implantation. Such may be carried out
by contacting the endometrium with a neutralizing Fab
fragment specific for β_3. For purposes of the present
invention, Fab fragments from monoclonal antibodies which

30 bind β_3 are within the scope of the invention. Fab fragments
include, but are not limited to, Fab fragments from
monoclonal antibodies SSA6 and 23C6. The Fab fragment may
remain *in vivo* for a therapeutically effective time to
prevent embryo implantation. The Fab fragment comprises the

35 ligand binding portion of a monoclonal antibody for β_3, i.e.
the binding site for β_3. A neutralized Fab fragment is used

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in place of a typical monoclonal antibody to reduce the possibility of an inflammatory reaction.

Contraception is a further embodiment of the invention. A contraceptive may include a therapeutically
5 effective amount of neutralizing Fab fragment monoclonal antibodies specific for β_3 integrin in a pharmaceutically acceptable carrier, preferably adapted for intrauterine application. The Fab fragment may be from a monoclonal antibody which recognizes β_3, such as and not limited to
10 SSA6. The contraceptive may include β_3 in combination with other integrin subunits, such as and not limited to α_v for example. In the case of $\alpha_v\beta_3$, the Fab may be from monoclonal antibody 23C6.

Compounds which specifically block binding of the
15 embryo to this α_v/β_3 receptor are also included within the scope of the present invention. Examples include peptides containing the amino acid sequence arginine - glycine - aspartic acid, RGD (Pierschbacher et al., "Synthetic Peptide with Cell Attachment Activity of Fibronectin", *PNAS* **1983**,
20 *80*:1224) which is the active binding site for the vitronectin receptor. This sequence has been reported to block attachment of pregnancy derived cells (trophoblast) *in vitro* by researchers, Kao et al., "The Human Villous Cytotrophoblast: Interactions with Extracellular Matrix
25 Proteins, Endocrine Function, and Cytoplasmic Differentiation in the Absence of Syncytium Formation", *Development* **1988**, *130*:693. Thus, a contraceptive containing the sequence RGD may be administered locally to prevent embryo implantation.

Pharmaceutically acceptable carriers include, and
30 are not limited to, vaginal suppositories, intrauterine devices (IUD), gels such as slow release formulation, for example, depo forms of hormones - microcrystals injected and slowly released into the systemic circulation or delivered in silastic tubing. Contraceptive formulations would be
35 administered in about 10 μg/ml.

Methods of monitoring endometrial maturation is also within the scope of the present invention. The

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endometrium may be monitored for embryo receptivity, embryo implantation, infertility, endometrial replenishment and ovulation.

5   Diagnostic kits are also within the scope of this invention. Such kits include monoclonal antibodies to rapidly detect β_3 in solution; an absorbant detection device which contains pre-absorbed antibody against β_3 and to which uterine washings can be applied; a developer to make β_3 visible when present.

10   Compounds which prevent β_3 expression are also included within scope of present invention. Although the regulatory signal for the induction of the endometrial integrin on day 19 to 20 is not yet secured, as a result of the present invention, peritoneal fluid is now known to
15 contain bioactive agents which appear to be related to defective integrin expression in women with infertility and endometriosis. Bioactive components with an abnormal peritoneal milieu may lead to a breakdown in the paracrine crosstalk between endometrial cells within the uterus.
20 Candidates include interleukin-1β receptor antagonist, elevated in women with minimal and mild endometriosis and recently demonstrated to interfere with implantation in a rodent model, Simon, C., et al., Annual Meeting of the American Fertility Society, Montreal, S2-Abst O-3, 1993.
25 Open or unobstructed fallopian tube may be a prerequisite to endometriosis-mediated effects of endometrial β_3 integrin.

        In accordance with the present invention, the hydrosalphinx fluid is herein identified as a potential candidate for causing endometrial defects. The methods of
30 the present invention determined that over 50% of women with distal occlusion of the fallopian tube, which causes tubal fluid accumulation, were found to have a loss of the $\alpha_v\beta_3$ integrin during the time of implantation. Three women who were β_3 negative had complete correction of the defect of
35 integrin expression. These three women underwent endometrial biopsy before and after the hydrosalphinx was surgically removed. This suggests that fluid from the tube, which

accumulates and is allowed to empty back into the uterus
causes a dysfunction in the endometrium, resulting in
infertility. This is consistent with recent reports showing
a decrease in uterine receptivity in women with hydrosalphinx
5   compared to women without hydrosalphinx entering an IVF-*in
     vitro* fertilization clinic.

          The present invention is directed to a method of
     treating a mammal having endometriosis comprising screening a
     mammal suspected of having endometriosis for the presence of
10   hydrosalphinx fluid and inactivating said fluid. The step of
     inactivating the hydrosalphinx fluid includes, and is not
     limited to, removing the fluid or contacting antibodies to
     the fluid. Antibodies may include monoclonal, polyclonal,
     chimeric antibodies and Fab fragments. Alternatively,
15   screening for factors which may be isolated from
     hydrosalphinx fluid and inactivating said factor is
     contemplated by the present invention.

          The present is also directed to preventing embryo
     implantation by contacting fallopian tubes or the uterus with
20   hydrosalphinx fluid or hydrosalphinx fluid factors.
     Hydrosalphinx may remain *in vivo* for a therapeutically
     effective time to prevent embryo implantation.

          Contraception is a further embodiment of the
     invention. A contraceptive may include a therapeutically
25   effective amount of hydrosalphinx fluid, or factors isolated
     therefrom. A contraceptive may alternatively be introduced
     together with a pharmaceutically acceptable carrier. The
     dosage of a contraceptive of the present invention is
     determined with regard to weight, and clinical condition of
30   the patient. The proportional ratio of active ingredient to
     carrier will naturally depend on the chemical nature,
     solubility, and stability of the compounds, as well as the
     dosage contemplated. Contraceptives of the present invention
     are preferably adapted for fallopian tube or intrauterine
35   application.

The present invention is further described in the
following examples. These examples are not to be construed
as limiting the scope of the appended claims.

EXAMPLES

5 **Human Samples**

Endometrium was obtained from 35 reproductive age
women at the time of hysterectomy. Tissue was obtained from
the early proliferative (day 5) through late secretory phase
(day 28) and all hysterectomies were performed for benign

10 disease. Endometrial biopsies were performed on women as
part of their evaluation for infertility. All patients were
cycling normally and none had received hormones for at least
3 months prior to surgery. Dating of the endometrium was
assessed according to the criteria of Noyes et al., "Dating

15 the Endometrium", *Fertil. Steril.* **1950**, *1*:3. Endometrial
biopsies were evaluated in the context of timing of ovulation
and/or the onset of the next menstrual period. Samples were
judged as "out of phase" if histologic dating was delayed by
3 or more days relative to the predicted day of the menstrual

20 cycle. Proliferative endometrium was categorized based on
histology and on last menstrual period. Samples were
transported on ice to the laboratory and were snap frozen on
dry ice and stored at -70°C.

Antibodies

25 Monoclonal antibodies (Mabs) P1H5, P1B5, P1D6
specific to α_2, α_3, α_5 subunits, respectively, were acquired
from Drs. Elizabeth Wayner and William Carter. Mabs TS2/7
and B-5H10 directed against the α_1 and α_4 subunits,
respectively were acquired from Dr. Martin Hemler. GoH3, a

30 specific Mab directed against α_6 was acquired from Dr. Arnoud
Sonnenberg. Mab SSA6 specific to the β_3 subunit was acquired
from Drs. Joel Bennett and James Hoxie. Mab LM142 against α_v
was acquired from Dr. David Cheresh. The β_4 antibody was
acquired from Dr. Steven Kennel. The 23C6 antibody which

35 recognizes β_3 attached to α_v was obtained from Michael
Horton.

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Immunohistochemistry

Immunoperoxidase staining was performed on cryostat
sections of endometrium samples from throughout the menstrual
cycle. Serial cryosections 4-8μ thick were placed onto
5 poly-L lysine coated slides, fixed in -20°C acetone for 10
minutes, and stained using Vectastain Elite® ABC kits (Vector
Laboratories, Burlingame, CA). Diaminobenzadine (DAB; Sigma
Chemical Co., St. Louis, MO.) was used as the chromagen.
Primary antibody was placed on cryosections following
10 blocking with 1% bovine serum albumin in PBS, and allowed to
bind at room temperature for 1 hour. A phosphate buffered
saline (PBS) pH 7.2 to 7.4 rinse was followed by secondary
antibody consisting of biotinylated goat anti-mouse antibody
for 30 minutes. Following a PBS rinse, the endogenous
15 peroxidases were quenched with a 30 minute incubation with
0.3% H_2O_2 in absolute ethanol, followed by a 30 minute
rehydration in PBS. Avidin:biotinylated horseradish
peroxidase macromolecular complex (ABC) was then incubated on
the sections for 30 minutes before adding diaminobenzadine
20 for 3 minutes to complete the reaction. Some samples were
treated with 1:200 dilution of fluorescein-labeled anti-mouse
antibodies for 1 hr., for immunofluorescent microscopy.
Samples were subsequently washed in PBS and mounted. The
resulting staining was evaluated on a Nikon microscope at low
25 (100x) and higher (400X) magnification with or without
fluorescence. Staining was judged as absent (-), weak ($\pm$),
moderate (+) or strong (++). Examples of each is presented
in Figure 3. Photomicrographs were made using Kodak T-MAX
100 ASA film.

30 Integrin Distribution in Normal Endothelium

The distribution of α_2, α_3, α_6, and β_4 subunits of
integrins which recognize primarily collagen (Col) and/or
laminin (LM) is shown in Figure 1 A-D. These subunits were
present on glandular epithelium ($\leftarrow$) throughout the menstrual
35 cycle. Their distribution within the endometrium was typical
of that seen for most epithelial tissues. The α_2 and α_3

subunits were distributed around the entire circumference of the cells, while the α_6 and β_4 subunits appeared to be localized at the basolateral surface, adjacent to the basement membrane (BM) of the endometrial glands. The

5   expression of these subunits by the mesenchyme (*) was less pronounced. While moderate staining was seen for α_6 on stromal cells (Figure 1C) very little staining was noted for β_4. The expression of the α_4 and α_5 subunits of integrins known to bind fibronectin (Table 1) was quite restricted.

10   The α_4 subunit was undetectable above background staining (compare Figure 1E with Figure 2C) in either epithelium or mesenchyme. The α_5 subunit (Figure 1F), representative of the classic fibronectin receptor was not seen on the epithelial components, but was strongly expressed in the

15   mesenchyme which is rich in fibronectin.

TABLE 1
DISTRIBUTION OF INTEGRINS BY LIGAND SPECIFICITY

| | Ligands | |
	Collagen/Laminin	Fibronectin/Vitronectin
	α_1/β_1	α_4/β_1
Integrin Subunit	α_2/β_1	α_5/β_1
	α_3/β_1	α_v/β_1
	α_6/β_1	α_v/β_3
	α_6/β_4	

25   The intensity of immunostaining for three other subunits of integrins was found to change in a menstrual cycle-dependent manner. Immunostaining for α_1 in the proliferative phase (Figure 2A) was only slightly above background levels (Figure 2C). The intensity of staining

30   increased throughout the secretory phase (Figure 2B). This intense circumferential staining was found on glandular and luminal epithelium on all samples from day 15 to 28. Likewise, α_v was weakly expressed on both the epithelium and

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mesenchyme in the proliferative phase (Figure 3A) and
staining increased gradually during the secretory phase to
the level noted in Figure 3B. During the proliferative phase
β_3 staining was only present on the mesenchymal cells (Figure

5   3C). Increased β_3 staining was apparent on the endometrial
epithelium only after day 19 of the menstrual cycle (Figure
3D) on the luminal as well as glandular epithelium, and was
also present in a pericellular distribution. In contrast,
the basalis layer did not significantly stain for either α_v

10   or β_3. This changing pattern of epithelial α_v and β_3
throughout the cycle was studied in 35 endometrial samples
and is depicted graphically in Figure 4A and 4B.

Collagen/laminin receptors (col/LM) characterized
by α_2, α_3 and α_6 were uniformly expressed throughout the

15   menstrual cycle, see Table 2. The pericellular distribution
of α_2 and α_3 subunits was distinctly different from that of α_6
subunit. Characteristic of a laminin receptor, α_6 was
concentrated on the basolateral surface. The β_4 subunit
which pairs with α_6, was also found distributed on the

20   basolateral surface of epithelial cells, and its distribution
appeared even more restricted to the basal pole. The α_5/β_1
integrin, a major fibronectin receptor, was also uniformly
expressed throughout the menstrual cycle. Unlike the
collagen and laminin receptors, the distribution of α_5/β_1 was

25   limited to the mesenchyme.

The temporal pattern of distribution of α_v was
varied. Immunostaining was first detected prior to the
secretory phase with an increase in intensity throughout the
cycle. One subunit known to pair with α_v is β_3. β_3 is not

30   characteristically present on epithelial cells. The abrupt
appearance of the β_3 subunit after day 19 suggests that
expression of the vitronectin receptor is regulated in human
endometrium. The increased epithelial α_v/β_3 staining in
normal cycles correlates to an implantation window within the

35   secretory phase. While the physiologic basis for the
implantation window has not been previously established, a
proposed role of integrins in the initial interaction between

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maternal and embryonic cells indicates an endometrial period of receptivity.

TABLE 2

**DISTRIBUTION OF INTEGRIN SUBUNITS IN NORMAL ENDOMETRIUM
DURING THE MENSTRUAL CYCLE**

Cell Type	Col/LM					FN/VN			
	α_1	α_2	α_3	α_6	β_4	α_4	α_5	α_v	β_3
Epithelial proliferative	O	●	●	●[b]	●[b]	O	O	O	O
early secretory	●	●	●	●	●	O	O	*	O
late secretory	●	●	●	●	●	O	O	●	●
Stromal proliferative	O	O	O	●	O	O	●	●	●
early secretory	O	O	O	*	O	O	*	●	●
late secretory	*	O	O	●	O	O	*	●	●

b = basolateral distribution of staining
● = + or ++ staining
* = ± staining
O = - staining

Integrins in Discordant Endometrium

The presence of the epithelial β_3 subunit appeared to be a consistent internal marker of luteal phase maturation, and the timing of β_3 expression correlated with the peri-implantation period or window of embryo implantation. To investigate whether this phenomenon would be useful in the clinical evaluation of endometrial biopsies, immunostaining was performed on luteal phase endometrial samples from cycles which showed evidence of maturational delay. Endometrial biopsies from 25 women who had concordance of menstrual and histologic dating ("Normal" group) were compared to 12 biopsies which were identified as ≥ 3 days out of phase (OOP) based on either the time of ovulatory or the subsequent menses. Samples were immunostained for α_1, α_v and β_3 subunits. All biopsies were

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performed on days 20 to 24 of the menstrual cycle. In all
instances, immunostaining for these three antigens was
present on endometrial epithelia from the normal group. In
biopsies which were delayed by 3 days or more, α_1 and α_v
5   staining was present, but epithelial β_3 staining was absent.
The comparison of β_3 staining intensity in the two groups is
shown in Figure 5A. Accompanying photomicrographs of β_3
immunostaining from out of phase biopsies (OOP; B) and normal
"in phase" biopsies (C) is included, which demonstrates the
10 discrepancy seen in β_3 staining. In subsequent treatment
cycles, 2 OOP patients underwent repeat biopsy during a
normalized cycle at which time immunostaining for epithelial
β_3 was present. This suggests that the lack of β_3 was not an
intrinsic defect in the OOP group. Rather, the discordant
15 biopsies which lacked β_3 had not yet established the
mid-luteal phenotype of normal day 20 to 24 endometrium.

Cell Harvest and NP-40 Extraction
            To further demonstrate that immunohistochemical
staining accurately reflected changes in the expression of β_3
20 subunit on endometrial epithelium, immunoblots (Western
blots) were performed on samples of enriched endometrial
glandular elements from proliferative and secretory phase.
Four samples of endometrium were obtained for the evaluation
of the β_3 subunit in proliferative (n = 2) and late secretory
25 (n = 2) endometrial epithelium. Each sample was placed in
Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis,
MO), supplemented with 10% fetal bovine serum (Flow
Laboratories, McLean, VA) glucose (4500 mg/L), Hepes buffer
(25 mM), L-glutamine (584 mg/L), and sodium bicarbonate (3.7
30 gms/L). Endometrium was minced in a plastic petri dish prior
to incubation with 6 mg of collagenase (type 1A, 550
units/mg; Sigma, St Louis, MO) for 2 hours at 37°C utilizing
modifications of the procedures described by Satyaswaroop et
al. in "Isolation and Culture of Human Endometrial Glands",
35 *J. Clin. Endocr. Metab.* **1979**, *48*:639. The resulting
suspension was successively passed through a 250 μm sieve and

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a 38 μm sieve (Newark Wire Cloth Co, Newark NJ). The course
(250 μm) sieve removed undigested material, while the second
retained the glandular elements and excluded the individual
stromal and blood cells. After thorough rinsing, the
5   glandular elements were obtained by backwashing with 10 to 20
ml of DMEM. The isolated glandular structures were then
transferred to a 1.5 ml microfuge tube and centrifuged 3
times (82 x g) for 2 minutes with intermittent washes with
PBS. Membrane extracts were prepared by adding small volumes
10 (100-200 μl) of 10 mM Tris-acetate, pH 8.0, 0.5% NP-40, 0.5
mM Ca^{2+} (TNC) with 2 mM PMSF (phenyl methyl-sulfonyl
fluoride) to the final pellet, pipetted and incubated on ice
for 15 minutes. The lysate was centrifuged for 5 minutes at
16,000 x g in a microcentrifuge. The resulting supernatant
15 was called NP-40 extract and was frozen at -70°C until use. A
portion of the original, undigested tissue was cryosectioned
for immunohistochemical localization of β_3.

Gel Electrophoresis and Immunoblots

The protein concentration of each NP-40 extract and
20 an extract of platelets (positive control) was determined
using technique described by Lowry et al., "Protein
Measurement with Folin Phenol Reagent", *J. Biol. Chem.* **1951**,
193:265. Samples with equal amounts of protein were added to
electrophoresis sample buffer (62.5 mM Tris base, 2% SDS, 10%
25 glycerol, pH 6.8). Samples were analyzed by SDS-PAGE using
6% polyacrylamide gels, using non-reducing conditions
described by Laemmli, U.K., "Cleavage of Structural Proteins
During Assembly of the Head of Bacteriophage T4", *Nature*
1970, *227*:680. The gel was transferred to nitrocellulose
30 using a Biorad transfer apparatus (Biorad, Richmond, CA) and
blocked with 4% BSA in PBS with 0.2% Na Azide for 1 hour.
After addition of primary antibody (SSA6 supernatant) for 2
hours, the gels were developed using an alkaline
phosphatase-conjugated secondary antibody (Promega Corp.,
35 Madison, WI) according to methods described by Albelda et

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al., "EndoCAM:A Novel Endothelial Cell-Cell Adhesion
Molecule", *J. Cell Biol.* **1990**, *110*:1227.

As shown in Figure 6A, proliferative phase
epithelial structures had little to no immunostaining at 95
5   kD (lanes 2 and 3), compared to the positive control
(platelet extract; lane 1) or to samples from the secretory
phase (lanes 4 and 5) which showed strong staining for β_3.
The isolated endometrial glands appeared as tubular
structures free of surrounding stroma (Fig. 6B).
10   Immunofluorescent staining for β_3 from samples corresponding
to lanes 3 and 4 (mid proliferative phase and day 23,
respectively) are shown in Fig. 6C and D. Note the absence
of glandular staining in the proliferative sample, while both
glandular and luminal immunostaining is obvious from the
15   secretory phase. These data confirm that the expression of
epithelial β_3 in human endometrium is a cycle specific
phenomenon.

Determination of Endometriosis
Patient Selection
20   Biopsies were obtained from throughout the
menstrual cycle from 500 women undergoing evaluation for
infertility at the University of Pennsylvania. The biopsies
were screened for participation in the study based on
criteria including accurate knowledge of menstrual cycle day
25   or "in phase" histology and freedom from menstrual cycle
disturbances (anovulation (suspension or cessation of
ovulation), perimenopausal state (dysfunctional uterine
bleeding), luteal phase defect (histologic evidence of
maturational delay of the endometrium), the presence or use
30   of an intrauterine device or endometritis (inflammation of
endometrium due to infec   us agents or chemical irritation).
Patients with endometrial hyperplasia (an increase in the
number of cells in endometrium), neoplasia (a pathologic
process resulting in formation or growth of abnormal tissue
35   or tumors, benign or malignant), and those on hormonal
therapy (including oral contraceptives, estrogen replacement

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therapy and progestens) were likewise excluded. Two hundred
and sixty eight women were eligible for enrollment in this
study, ranging from cycle day 2 through 27. The most common
primary diagnosis was endometriosis (41.4%) followed by
5 unexplained infertility (23.5%), see Table 3. Due to the
participation criterion of normal "in phase" histology as set
forth above, all cases of luteal phase deficiency were
excluded.

Table 3
10   **Diagnoses of Subjects with Infertility**
                  Undergoing Endometrial Biopsy (N=268)

Diagnosis	Number (%)
endometriosis	111 (41.4)
tubal disease	38 (14.2)
pelvic adhesions	17 (6.3)
male factor	18 (6.7)
fibroids	9 (3.4)
habitual pregnancy loss	6 (2.2)
in utero DES exposure	4 (1.5)
hyperprolactinemia	2 (0.7)
unexplained infertility	63 (23.5)

Endometrial biopsies of 241 patients were obtained
after day 19 (post ovulatory day 6 or greater) based on
25 urinary luteinizing hormone, LH, surge or next menstrual
period, a time in the menstrual cycle when epithelial β_3 is
expressed, Lessey, et al., *supra*. The remaining patients
(n=27) were biopsied prior to day 20 and therefore would not
be expected to produce the β_3 integrin. Based on routine
30 infertility workup, including diagnostic laparoscopy which
involves the examination of the contents of peritoneum with a
laparoscope passed through the abdominal wall, 105 (43.6% of
the group) of these women had endometriosis. The 105 women
were then considered as the test cases. Twenty women with
35 endometriosis also had documented prior fertility, previously
produced young, having parity ≥ 1, where each women had ≥ 1
prior birth, and had undergone bilateral tubal ligation or
had clearly identified male factor infertility, (or
infertility of the male partner). These were chosen to serve

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as fertile controls. The remaining 116 women (48.1% of the group) were considered the infertile controls.

Immunohistochemistry

Immunoperoxidase staining was performed on the 241
5   samples as described by Lessey, et al., *supra*. Briefly,
immunoperoxidase staining was performed on cryostat sections
of endometrium throughout the menstrual cycle. Serial
cryosections 4-8μ thick were placed onto poly-L lysine coated
slides, fixed in -20°C acetone for 10 minutes, and stained
10 using Vectastain Elite® ABC kits (Vector Laboratories,
Burlingame, CA). Diaminobenzadine (DAB; Sigma Chemical Co.,
St. Louis, MO.) was used as the chromogen. Primary antibody
consisting of SSA6 (specific to the β_3 subunit of the
vitronectin receptor) was placed on cryosections following
15 blocking with 1% bovine serum albumin in phosphate buffered
saline, PBS, and allowed to bind at room temperature for 1
hour. A PBS rinse, pH 7.2 to 7.4, was followed by secondary
antibody consisting of biotinylated goat anti-mouse antibody
for 30 minutes. Following PBS rinse, the endogenous
20 peroxidases were quenched with a 30 minute incubation with
0.3% H_2O_2 in absolute ethanol, followed by a 30 minute
rehydration in PBS. Avidin: biotinylated horseradish
peroxidase macromolecular complex (ABC) was then incubated on
the sections for 30 minutes before adding diaminobenzadine
25 for 3 minutes to complete the reaction. Samples were
subsequently washed in PBS and mounted.

Immunostaining for endometrial β_3 was performed on
each of the 268 endometrial biopsies obtained from throughout
the menstrual cycle (see Figure 7). Examples of cycle
30 specific expression of β_3 subunit using the
immunohistochemical staining are shown in Figure 8A
(proliferative), Figure 8B (day 18), Figure 8C (day 22) and a
sample from cycle day 23 with negative staining in a patient
with endometriosis whose "in phase" biopsy was read as "day
35 22-23" on histologic section (D). As seen in all cases,
immunostaining of the vascular endothelium remains present

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even in samples with no epithelial staining for this
integrin, thus serving as a positive internal control for the
immunostaining technique. Based on the distribution of β_3
shown in Figure 7 and the theory that the β_3 subunit is
5 normally expressed in endometrium from day 20 and later, all
samples which were histologically dated to cycle day 20 or
greater were included for further evaluation (n=241). Cases
with known endometriosis (102/241; 43.5%) were compared with
infertile controls without endometriosis (116/241; 48.1%) and
10 with 20 fertile controls (20/241; 83%). As listed in Table
4, both the endometriosis group and the infertile controls
were younger than the fertile controls. The resulting
staining was evaluated on a Nikon microscope at low (100X)
and higher (400X) magnification. The HSCORE, a semi-
15 quantitative index which estimates both intensity and
distribution of positive staining for β_3 integrin, was
calculated using the following equation:

$$HSCORE = \Sigma \, Pi \, (i + 1)$$

where i = intensity of staining with a value of 1, 2, or 3,
20 weak, moderate, or strong, respectively, and Pi is the
percentage of stained epithelial cells for each intensity,
varying from 0-100%. Low intraobserver values (r=0.994;
p=0.00001) in uterine tissues has been reported using this
technique by Budwit-Novotny, D.A., et al.,
25 "Immunohistochemical Analyses of Estrogen Receptor in
Endometrial Adenocarcinoma Using a Monoclonal Antibody",
Cancer Res., 1986 *46*:5419-5425. Examples of representative
HSCOREs reflecting changing levels of staining intensity and
distribution is illustrated Figure 8. Photomicrographs were
30 made using Kodak 100 ASA film.

Statistical Analysis

       Infertile endometriosis patients were compared to
infertile controls without endometriosis and fertile
controls. Comparisons were also made between nulliparous

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endometriosis patients and parous endometriosis patients on selected characteristics such as age and HSCORE and were performed using the nonparametric Wilcoxon text. Wilcoxon, F., Individual Comparisons by Ranking Methods. *Biometrics*
5 *Bull* 1945; 1:80-85.

        Receiver Operator Characteristic (ROC) analysis was used to determine the optimal cutoff value for HSCORE in women prospectively undergoing laparoscopy. Metz, C. E., Basic Principles of ROC analysis. Sem Nuclear Med 1978;
10 8:283-298. An HSCORE cutoff value was sought as the highest positive predictive value of endometriosis which meant seeking a high specificity at the cost of moderate sensitivity. A high positive predictive value minimizes the number of women who would otherwise undergo unnecessary
15 surgery, using this test as a diagnostic modality for endometriosis. The HSCORE cutoff value was 0.7, which yielded a sensitivity of 38%, specificity of 91%, and a positive predictive value of 86% for the detection of endometriosis.

20   The association between HSCORE and endometriosis was first evaluated by a nonparametric test (Wilcoxon test) and further evaluated by multiple logistic regression in the sample of 241 women whose biopsies were histologically cycle day 20 or greater. As seen in Table 4, the mean HSCORE was
25 significantly lower for the infertile endometriosis group (p < 0.003) compared to fertile controls (p < 0.002) and the infertile controls without endometriosis (p < 0.012; Table 4). Interestingly, the infertile controls without endometriosis also had a borderline lower HSCORE for β_3
30 staining than the fertile control group (p = 0.05).

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Table 4
Demographic and HSCORE Characteristics of Subjects Whose Endometrial Biopsies Were Histologically Normal and Cycle Day 20 or Greater

	Infertile w/ Endometriosis	Infertile w/o Endometriosis Infertile Controls	Fertile Controls
	n=105	n=116	n=20
Characteristic			
Age (yr)			
mean ± S.D.	34.1 ± 4.1[+]	34.5 ± 4.7[++]	36.9 ± 4.9
Severity			
AFS score			
I	40 (38.5%)		
II	44 (42.3%)		
III	15 (14.3%)		
IV	6 (5.8%)		
HSCORE			
Mean ± S.D.	1.30 (±1.09)[*]	1.68 (±0.95)[**]	2.15 ± (0.66)
Median	1.3	1.75	2.05
Max	3.6	3.4	3.6

[+] $p < 0.003$ vs. fertile controls, by Wilcoxon test.
[++] $p < 0.01$ vs. fertile controls, by Wilcoxon test.
[*] $p < 0.02$ vs. fertile controls and $p < 0.012$ versus infertile controls without endometriosis, by Wilcoxon test.
[**] $p = 0.05$ vs. fertile controls, by Wilcoxon test.

Severity of endometriosis based on AFS classification appeared to be associated with the presence or absence of the β_3 immunostaining. All 105 patients with infertility and endometriosis were stratified by severity of disease. The distribution of cases by AFS severity score is listed in Table 4. The HSCORE of the two groups based on AFS severity score, I and II (minimal and mild; n = 84) versus III and IV (moderate and severe; n = 21) demonstrated a significant difference using a Wilcoxon test (p = 0.0151). Therefore, defective β_3 expression is specifically associated with endometriosis stages I and II.

Multiple logistic regression using EGRET was used to evaluate the association between endometriosis and HSCORE, after adjusting for the effects of age and parity status, and taking into account the potential interaction of parity

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status. Mauritisen, R. EGRET. Statistics and Epidemiology
Research Corporation Software. Seattle, WA, 1990.

By multiple logistic analysis, the crude relative
risk (RR) and 95% confidence interval (CI) based on a model
5 with only HSCORE as the independent variable and the presence
or absence of endometriosis as the dependent (outcome)
variable, was 0.6 (0.5-0.8) when calculated for an HSCORE of
0.7 (by ROC analysis). A relative risk (RR) of 1 means there
is no effect, that two groups are the same with regard to
10 risk for endometriosis. A RR of 0.6 is highly significant,
confidence intervals show the range of confidence of the
test. Together, RR and CI define an increased risk of having
endometriosis in nulliparous women with a HSCORE of 0.7 or
less. The addition of age into the model did not change the
15 result, but there was a statistically significant interaction
with parity status (p = 0.030). Accordingly, the adjusted RR
(95% CI) was 0.6 (0.4-0.9) for nulliparous women compared to
1.2 (0.8-1.8) for parous women. These results indicate that
the absence of β_3 (as indicated by HSCORE) in nulliparous
20 women is a statistically significant prediction of
endometriosis. Restricting the analysis to women with only
minimal and mild disease did not change the result
substantially. The adjusted RR (95% CI) in nulliparous women
versus 0.5 (0.4-0.8) compared to 1.2 (0.7-1.8) in parous
25 women.

To further examine this relationship between
reproductive history and HSCORE, the 105 patients with
endometriosis were stratified by parity and compared to the
fertile control group (Table 5). The control population was
30 older than the parous and nulliparous endometriosis groups.
The HSCORE in the nulliparous endometriosis group was less
than that of the parous endometriosis group and the fertile
control group (p < 0.0001, for each group), but there was no
difference between the parous group with endometriosis and
35 the fertile controls (p = 0.98). The comparison between
these three groups is depicted graphically in Figure 9.

Table 5
Comparison between Nulliparous and Parous Women
with Endometriosis and Normal Fertile Controls

Endometriosis Patients
n=105

	Nulliparous	Parous	Fertile Controls
	n=78	n=27	n=20

Characteristic

Age (yr)
| mean ± S.D. | 34.1 ± 4.1[+] | 34.3 ± 4.0[++] | 36.9 ± 4.9 |

Severity
AFS score
I	30 (38.5%)	10 (37.0%)	
II	33 (42.3%)	11 (40.7%)	
III	11 (14.1%)	4 (14.8%)	
IV	4 (5.1%)	2 (7.4%)	

HSCORE
Mean ± S.D.	1.03 (±1.04)[*]	2.10 (±0.79)[**]	2.15 ± (0.66)
Median	0.6	2.1	2.05
Max	3.6	3.6	3.6

[+] $p < 0.004$ vs. fertile controls, by Wilcoxon test.
[++] $p < 0.02$ vs. fertile controls, by Wilcoxon test.
[*] $p < 0.0001$ vs. parous endometriosis patients and
    $p < 0.0001$ versus fertile controls, by Wilcoxon test.
[**] $p < 0.98$ vs. fertile controls, by Wilcoxon test.

The observation that differences in integrin
expression correlated with the diagnosis of endometriosis,
prompted the evaluation of the usefulness of this marker in
predicting this disorder in women prospective to the
diagnostic laparoscopy. As a subgroup of the original 241
samples, 89 biopsies were obtained prospectively prior to
diagnostic laparoscopy. Chronological dating was established
by urinary luteinizing hormone, LH, surge and/or the onset of
the next menstrual period. Endometrial dating was performed
using the criteria of Noyes et al., "Dating the Endometrium",
Fertil. Steril. **1950**, *1*:3. All 89 of these women
subsequently underwent diagnostic laparoscopy to establish
the presence or absence of endometriosis. The severity of
the disease, when present, was staged according to the

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revised American Fertility Society, AFS, criteria set forth
in American Fertility Society: Revised American Fertility
Society classification of endometriosis, *supra*. The surgeons
performing the diagnostic laparoscopies were blinded with

5   regard to the result of the immunohistochemical findings.
Likewise the interpretation of the biopsy results (estimation
of β_3 expression) was done in a blinded fashion using HSCORE,
which estimates both intensity and distribution of positive
staining for β_3 integrin. The 0.7 HSCORE value, determined

10 as the optimal cutoff, yielded a sensitivity of 38%, a
specificity of 91% and the highest positive predictive value
of 86% for the prospective detection of endometriosis.
Overall, 50 patients were prospectively found at laparoscopy
to have endometriosis at a variety of AFS stages. Negative

15 staining, indicating no β_3 expression, based on ROC analysis,
was detected in 22 samples. Of these 22 patients without β_3
staining, 19 were found to have endometriosis. ROC analysis
was performed to establish the optimal cutoff value for
normal samples. To the best of Applicant's knowledge, the

20 present invention is the first non-surgical test that has
been demonstrated to predict the presence of minimal or mild
endometriosis.

All patients subsequently found to have
endometriosis were not missing the β_3 subunit. The present

25 invention identifies a subgroup of infertility patients with
a deficit in β_3 which characterizes women with defective
uterine receptivity. This association between endometriosis
and defective β_3 integrin expression may represent the first
cycle-specific defect during the putative window of

30 implantation. Detecting the loss of β_3 may signify patients
with endometriosis who are most at risk for infertility due
to an endometrial factor. While implantation may be the
result of a cascade of different molecules, the present
invention reveals the loss of a single component may be

35 sufficient to explain a failure of implantation.

The present invention reveals the loss of β_3,
normally expressed at the initiation of the window of

implantation, is associated with women who appear to be the
most infertile. Prospective use of this marker protein has
demonstrated that the β_3 integrin subunit is a useful test
with a high specificity and positive predictive value for the
5 nonsurgical diagnosis of minimal or mild endometriosis. As a
potential marker of uterine receptivity that may predict
fertility potential, the study of this subgroup of
infertility patients allows greater refinement of diagnosing
this enigmatic disease.

10 **Determination of Endometriosis in**
the Infertile Patient Population
        Biopsies will be obtained from throughout the
menstrual cycle from women being evaluated for infertility.
Women who are parous and nulliparous will be included in the
15 study. The biopsies will be screened for participation in a
study based upon criteria including accurate knowledge of
menstrual cycle day or "in phase" histology and freedom from
menstrual cycle disturbances (anovulation (suspension or
cessation of ovulation), perimenopausal state (dysfunctional
20 uterine bleeding), luteal phase defect (histologic evidence
of maturational delay of the endometrium), the presence or
use of an intrauterine device or endometritis (inflammation
of endometrium due to infectious agents or chemical
irritation). Patients with endometrial hyperplasia (an
25 increase in the number of cells in endometrium), neoplasia (a
pathologic process resulting in formation or growth of
abnormal tissue or tumors, benign or malignant), those on
hormonal therapy (including oral contraceptives, estrogen
replacement therapy and progestens), and those with luteal
30 phase deficiency will be excluded.
        Endometrial biopsies will be obtained after day 19
(post ovulatory day 6 or greater) based on urinary
luteinizing hormone, LH, surge or next menstrual period, a
time in the menstrual cycle when epithelial β_3 is expressed,
35 Lessey, et al., *supra*. Fertile and infertile controls will
also be selected.

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Immunoperoxidase staining will be performed on the
patient samples as described by Lessey, et al., *supra*.
Briefly, immunoperoxidase staining will be performed on
cryostat sections of endometrium throughout the menstrual
5 cycle. Serial cryosections 4-8μ thick will be placed onto
poly-L lysine coated slides, fixed in -20°C acetone for 10
minutes, and stained using Vectastain Elite® ABC kits (Vector
Laboratories, Burlingame, CA). Diaminobenzadine (DAB; Sigma
Chemical Co., St. Louis, MO.) will be used as the chromogen.
10 Primary antibody consisting of SSA6 (specific to the β_3
subunit of the vitronectin receptor) will be placed on
cryosections following blocking with 1% bovine serum albumin
in phosphate buffered saline, PBS, and allowed to bind at
room temperature for 1 hour. A PBS rinse, pH 7.2 to 7.4,
15 will be followed by secondary antibody consisting of
biotinylated goat anti-mouse antibody for 30 minutes.
Following PBS rinse, the endogenous peroxidases will be
quenched with a 30 minute incubation with 0.3% H_2O_2 in
absolute ethanol, followed by a 30 minute rehydration in PBS.
20 Avidin: biotinylated horseradish peroxidase macromolecular
complex (ABC) will then be incubated on the sections for 30
minutes before adding diaminobenzadine for 3 minutes to
complete the reaction. Samples will be subsequently washed
in PBS and mounted.
25   The HSCORE and statistical analysis, using Wilcoxon
test, ROC analysis, and p values, for infertile females is
expected to be similar to that of nulliparous infertile
females set forth above. HSCORE cutoff value, sensitivities
and specificities values will be determined for the detection
30 of endometriosis in infertile patients. The patients will
also be separated as to severity of endometriosis based on
AFS classification. EGRET will be used to evaluate the
association between endometriosis and HSCORE, after adjusting
for the effects of age and parity status, *supra*. Crude
35 relative risk and confidence intervals will be calculated for
endometriosis. Overall, it is expected that integrin
expression, detected by the absence of β_3, will correlate

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with the diagnosis of endometriosis in infertile females.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such
5 modifications are also intended to fall within the scope of the appended claims.

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WHAT IS CLAIMED:

1.   A method for predicting endometriosis comprising:

a.   obtaining a sample of endometrium selected from menstrual cycle day 20 to 24,

b.   identifying said sample of endometrium as nulliparous,

c.   contacting said sample with a monoclonal antibody specific for β_3 integrin,

d.   assaying for β_3 integrin in said sample, and

e.   correlating the absence of β_3 integrin expression with endometriosis, wherein said endometriosis is mild/minimal endometriosis.

2.   The method of claim 1 wherein said monoclonal antibody is SSA6.

3.   The method of claim 1 wherein said β_3 is combined with another integrin subunit.

4.   The method of claim 3 wherein said other integrin subunit is α_v.

5.   The method of claim 4 wherein said monoclonal antibody is 23C6.

6.   The method of claim 1 wherein said β_3 is detected by immunohistochemically staining said sample of endometrium contacted with said monoclonal antibody specific for β_3.

7.   The method of claim 1 wherein said β_3 is detected by immunoblotting β_3 from said sample of endometrium contacted with said monoclonal antibody specific for β_3.

8.   The method of claim 1 wherein said endometrium is human.

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9.   A method of using a monoclonal antibody to β_3 integrin to predict endometriosis comprising:

a.   obtaining a sample of endometrium selected from menstrual cycle day 20 to 24,

5   b.   identifying said sample of endometrium as nulliparous,

c.   contacting said sample with a monoclonal antibody specific for β_3 integrin,

d.   assaying for β_3 integrin in said sample, and

10   e.   correlating the absence of β_3 integrin expression with endometriosis, wherein said endometriosis is mild/minimal endometriosis.

10.   The method of claim 9 wherein said monoclonal antibody is SSA6.

15   11.   The method of claim 9 wherein said β_3 is combined with another integrin subunit.

12.   The method of claim 11 wherein said other integrin subunit is α_v.

13.   The method of claim 12 wherein said monoclonal

20   antibody is 23C6.

14.   The method of claim 9 wherein said endometrium is human.

15.   The method of claim 9 wherein said β_3 is monitored by immunohistochemically staining said sample of

25   endometrium contacted with said monoclonal antibody specific for β_3.

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16.   The method of claim 9 wherein said β_3 is monitored by immunoblotting β_3 from said sample of endometrium contacted with said monoclonal antibody specific for β_3.

5   17.   The method of claim 9 wherein said stages of the menstrual cycle are selected from the group consisting of follicular phase and secretory phase.

18.   A method of treating a mammal suspected of having endometriosis comprising screening said mammal 10   suspected of having endometriosis for the presence of hydrosalphinx fluid and inactivating said fluid.

19.   The method of claim 18 wherein inactivating said fluid is selected from the group consisting of removing said fluid and treating said fluid with antibodies to 15   hydrosalphinx fluid.

20.   A method of preventing embryo implantation comprising contacting fallopian tubes or the uterus with hydrosalphinx fluid.

21.   A contraceptive comprising a therapeutically 20   effective amount of hydrosalphinx fluid and a pharmaceutically accepable amount of a carrier.

FIG. IA

FIG. IB

FIG. IC

FIG. ID

FIG. IE
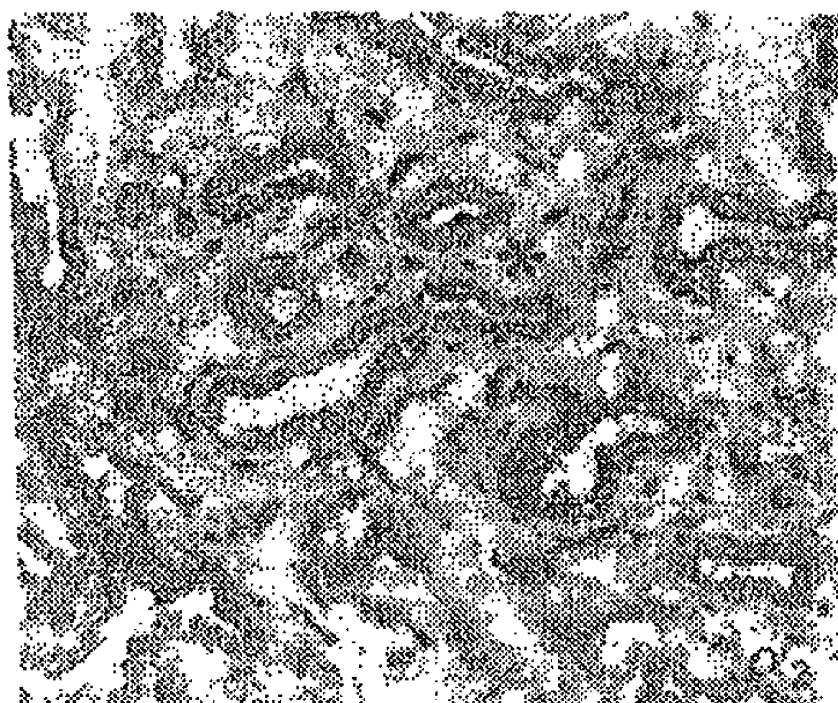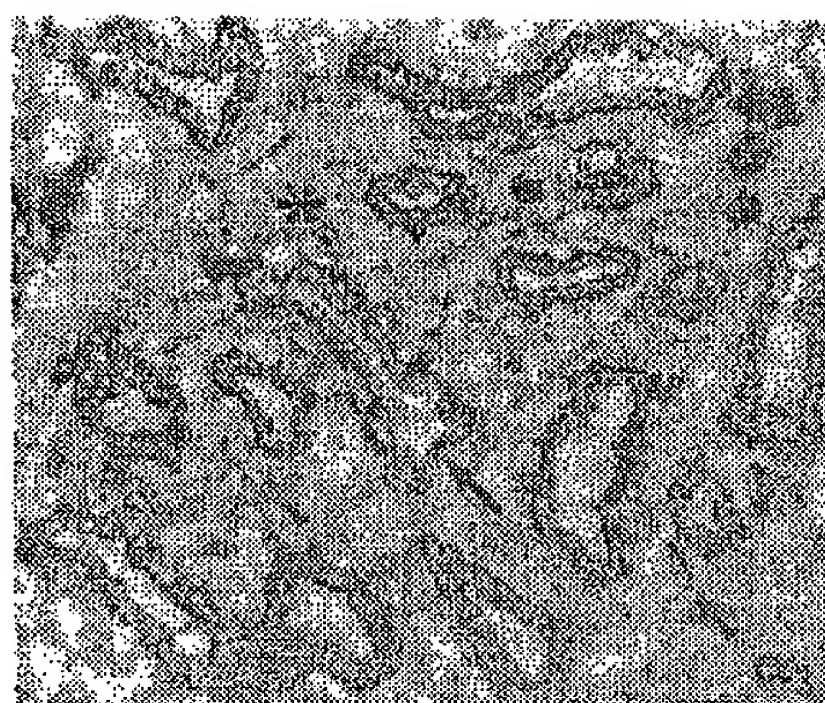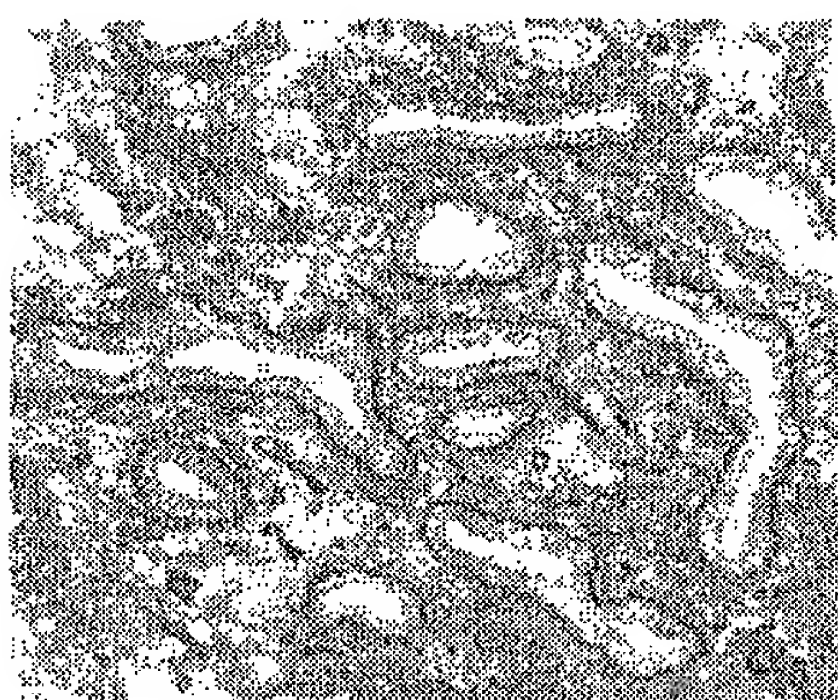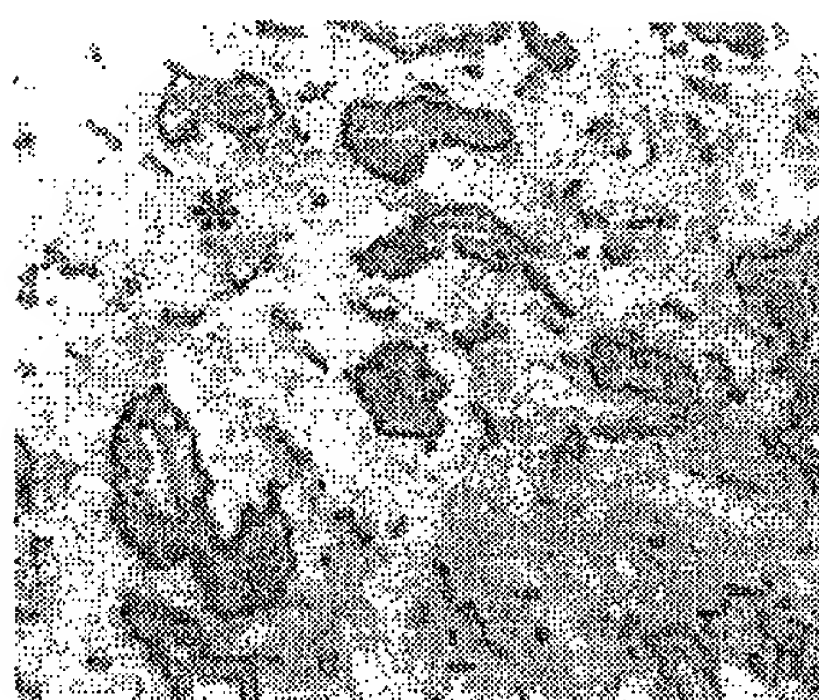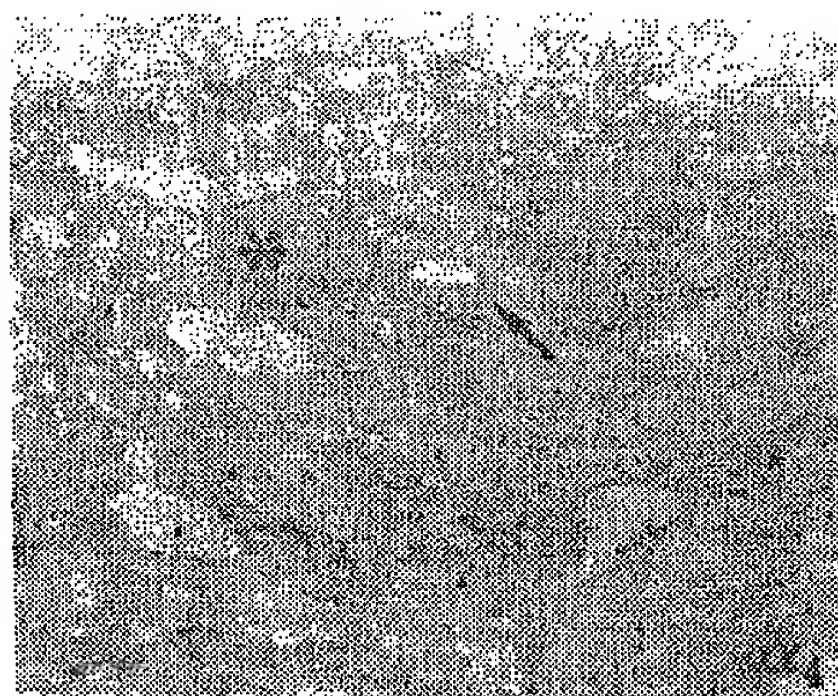
FIG. IF

FIG. 2A
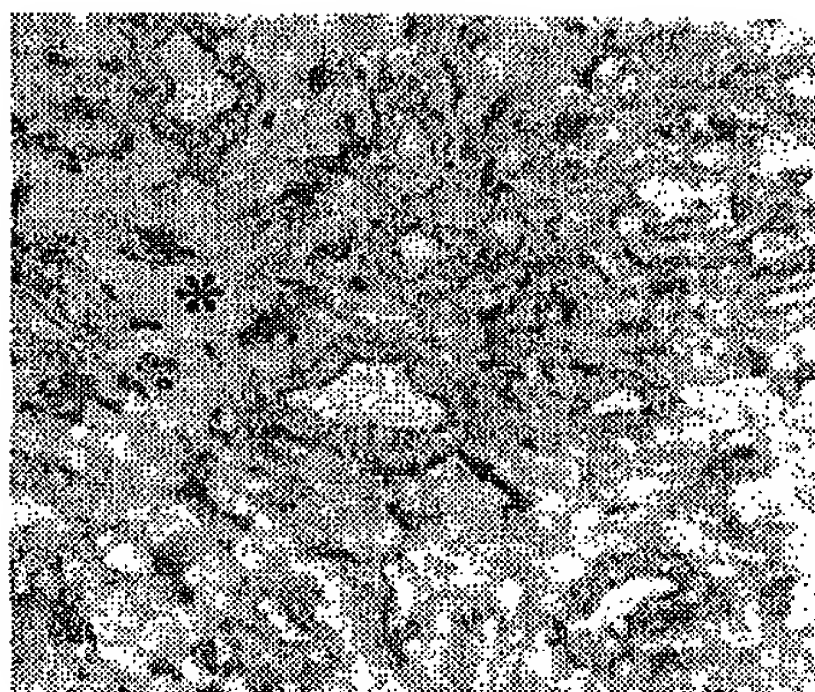
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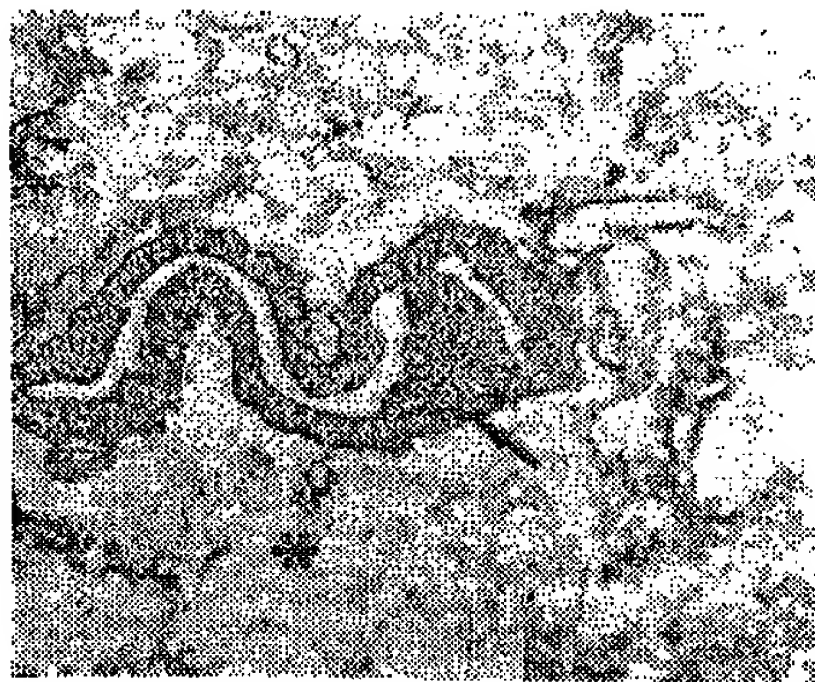
FIG. 2C
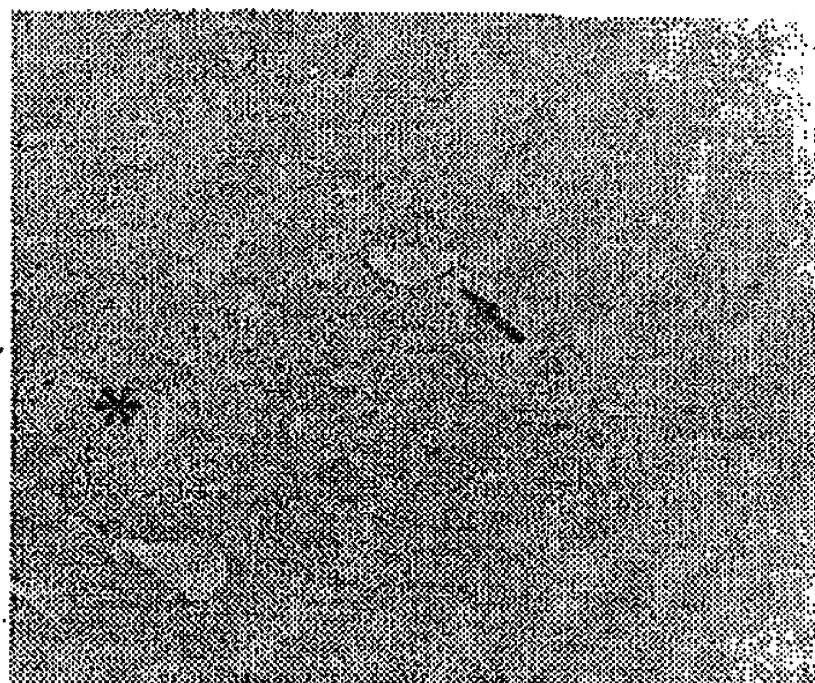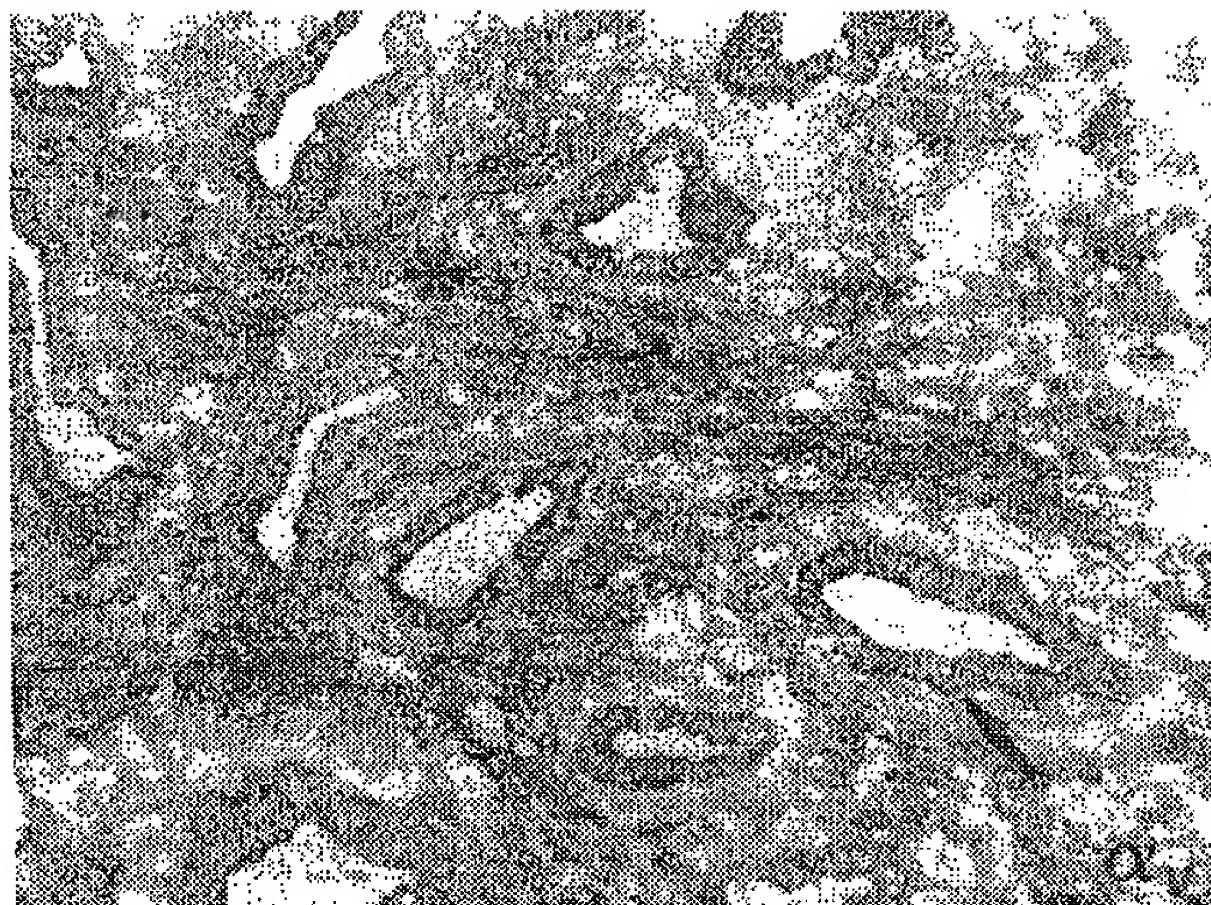
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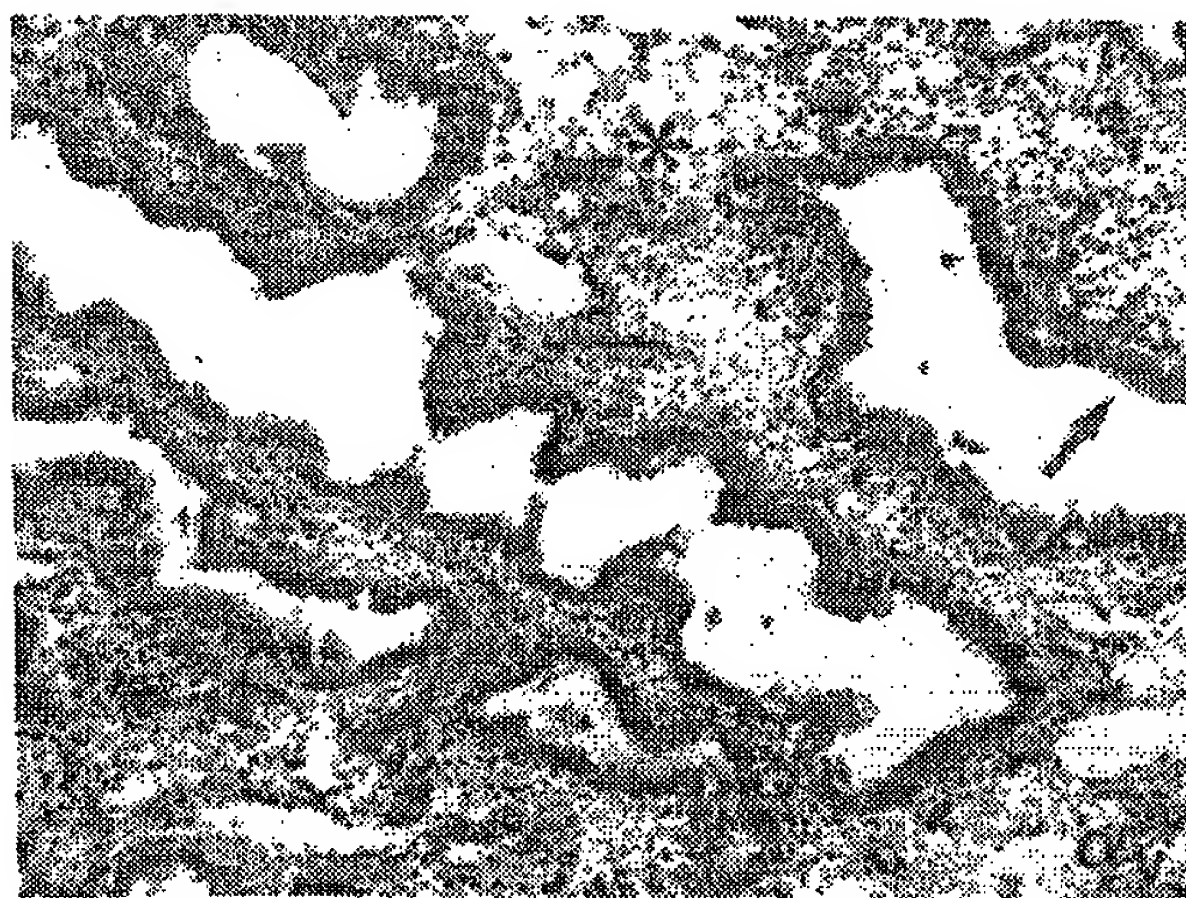
FIG. 3B
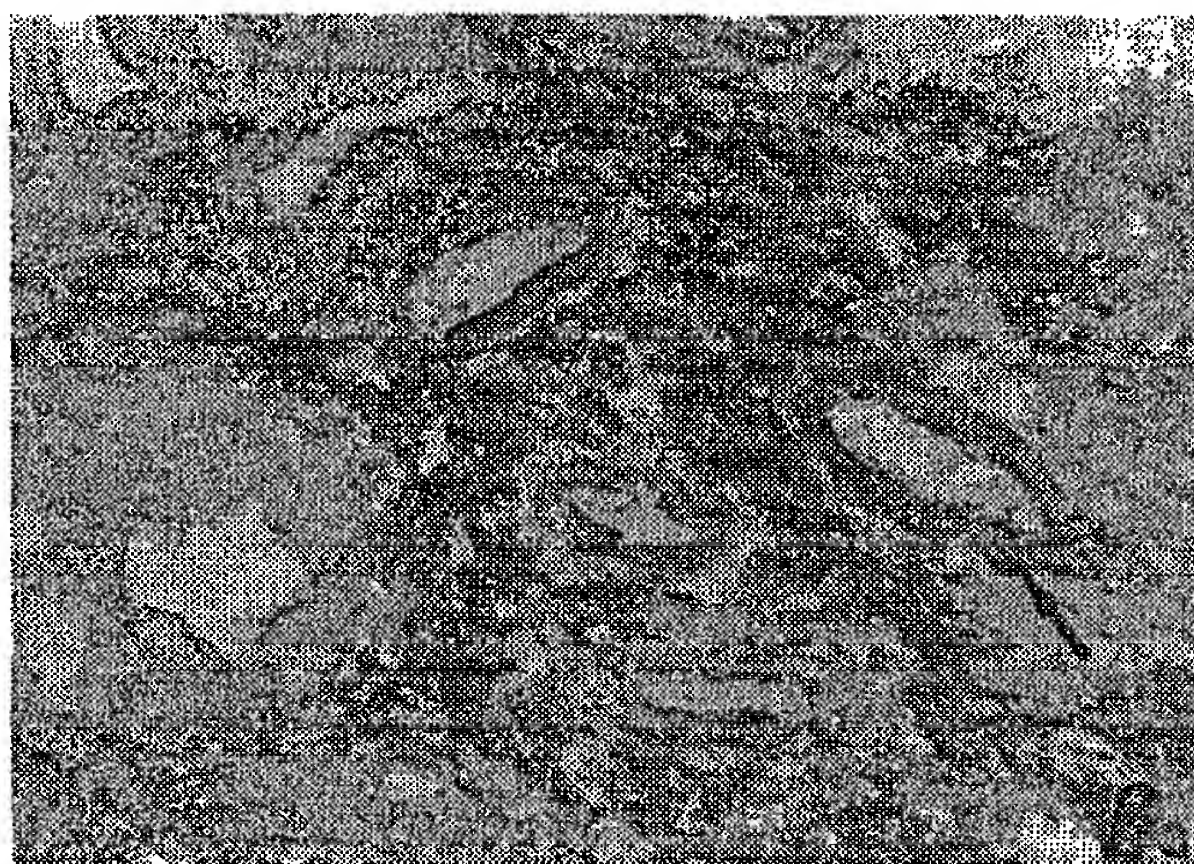
FIG. 3C
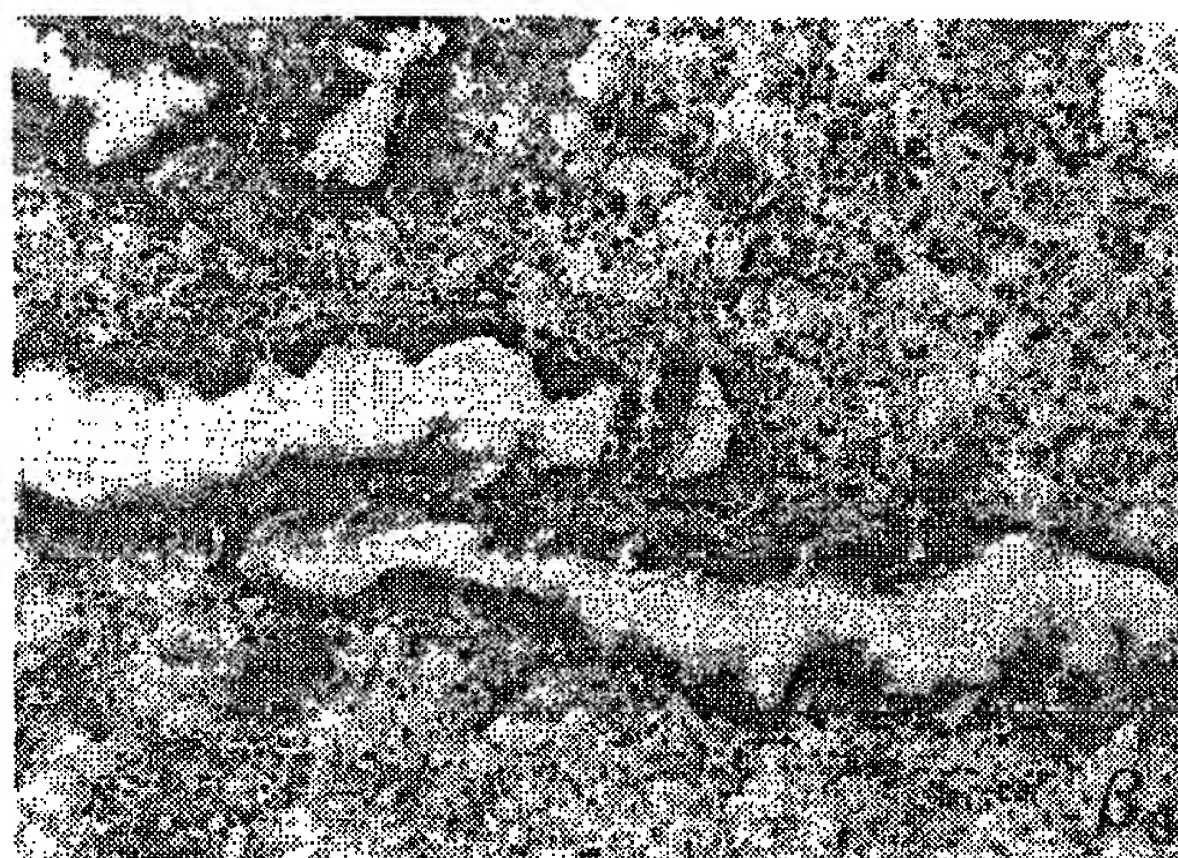
FIG. 3D
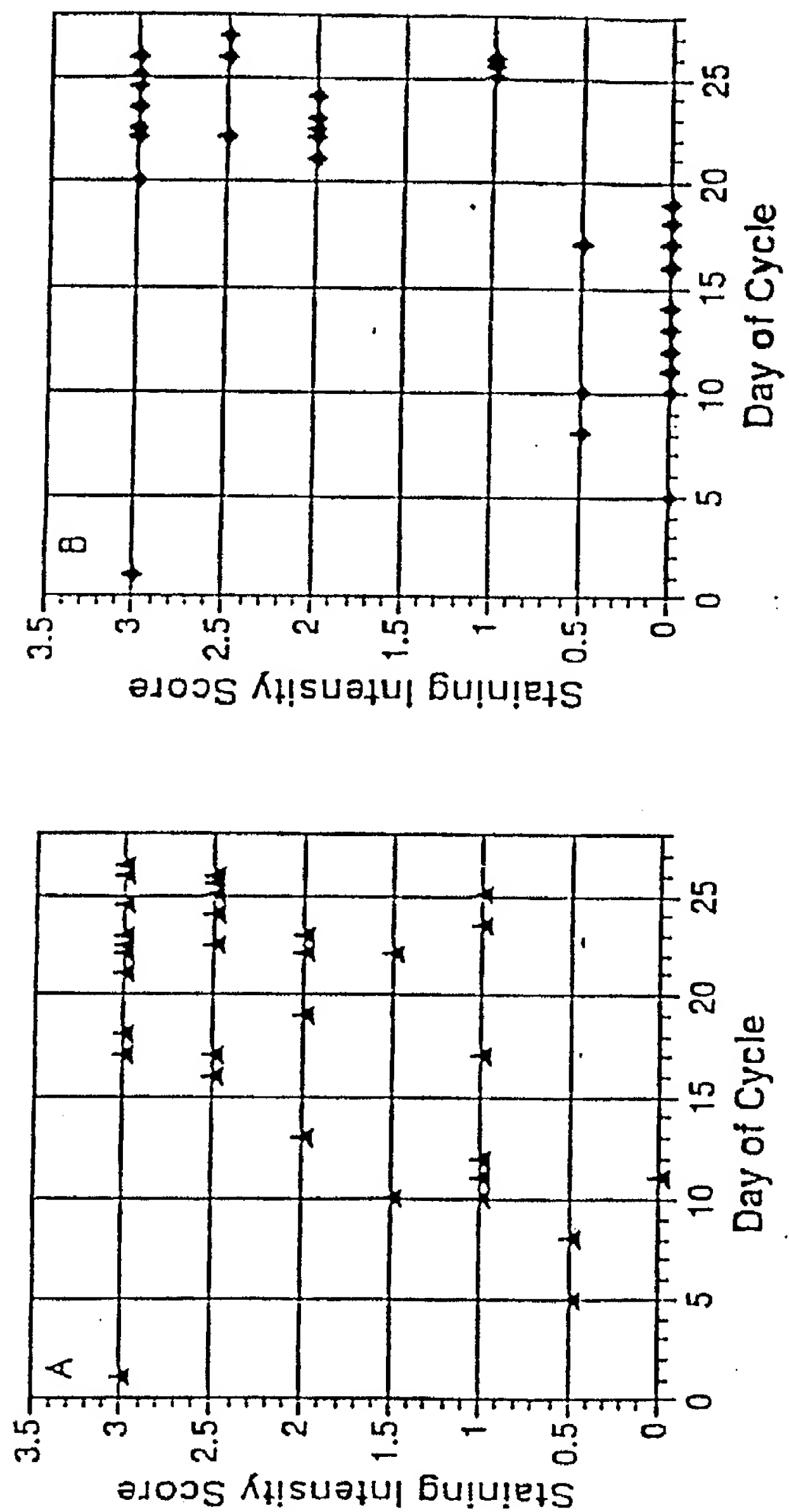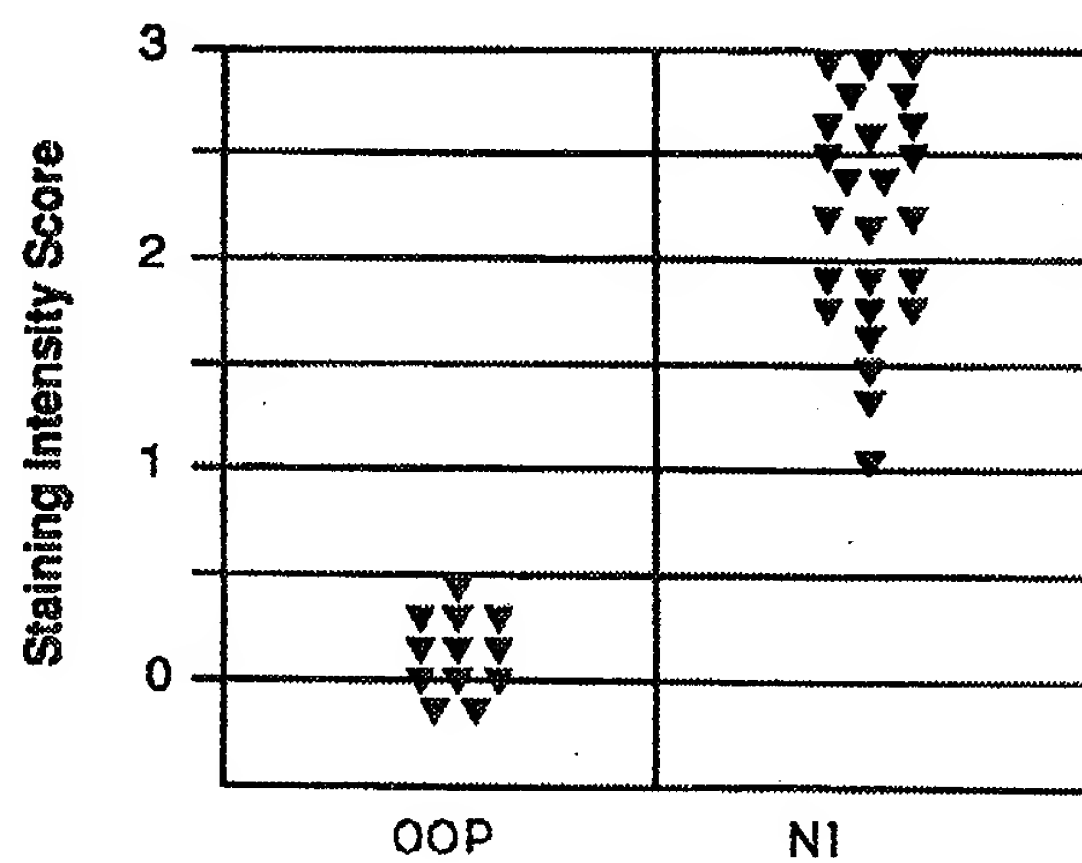
FIGURE 4

FIG. 5A

FIG. 5B
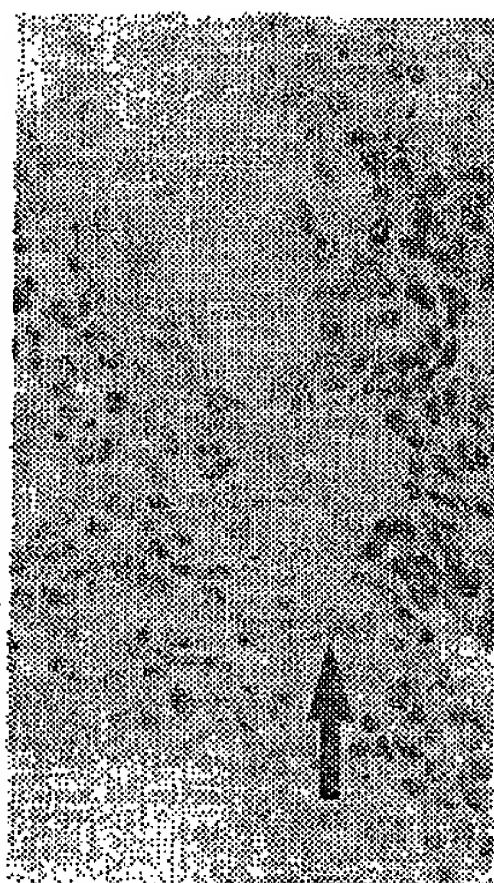
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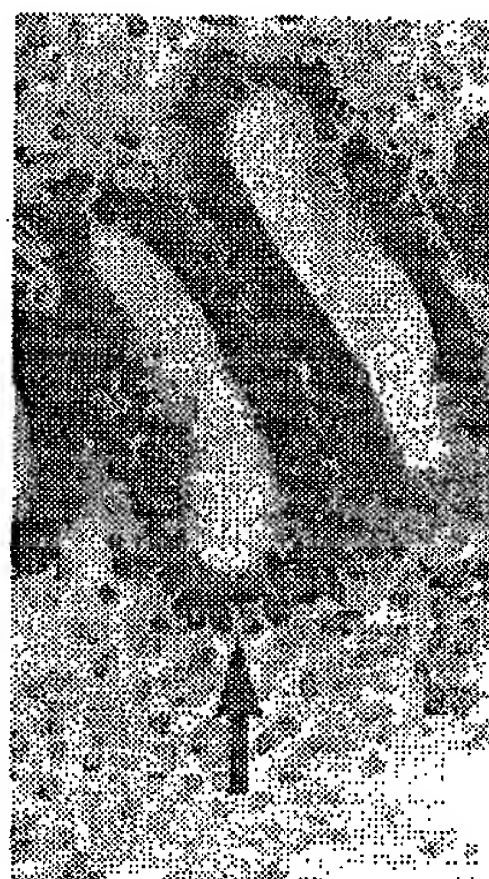
FIG. 6C
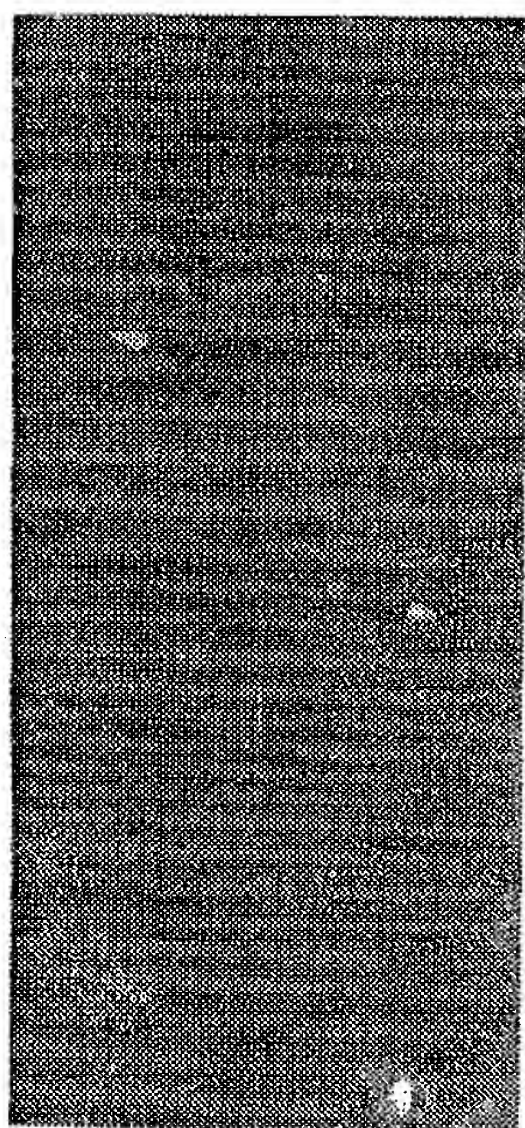
FIG. 6D
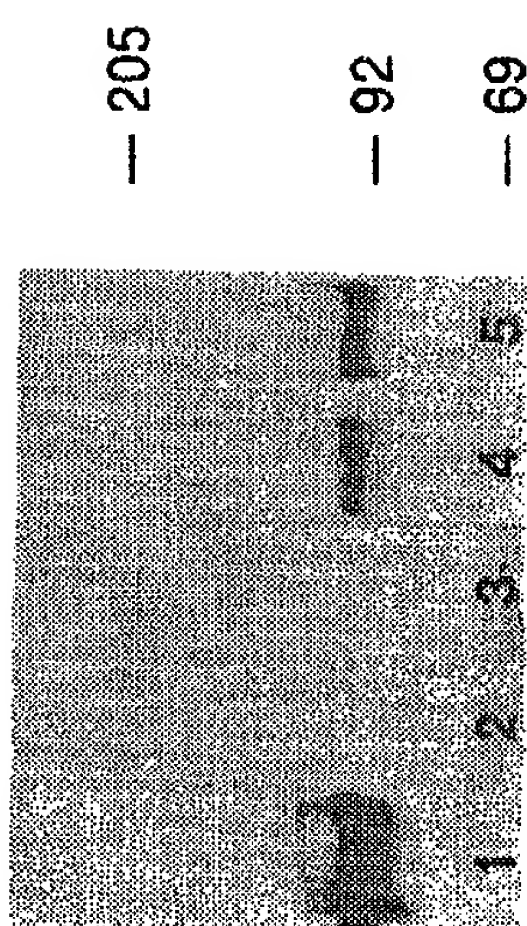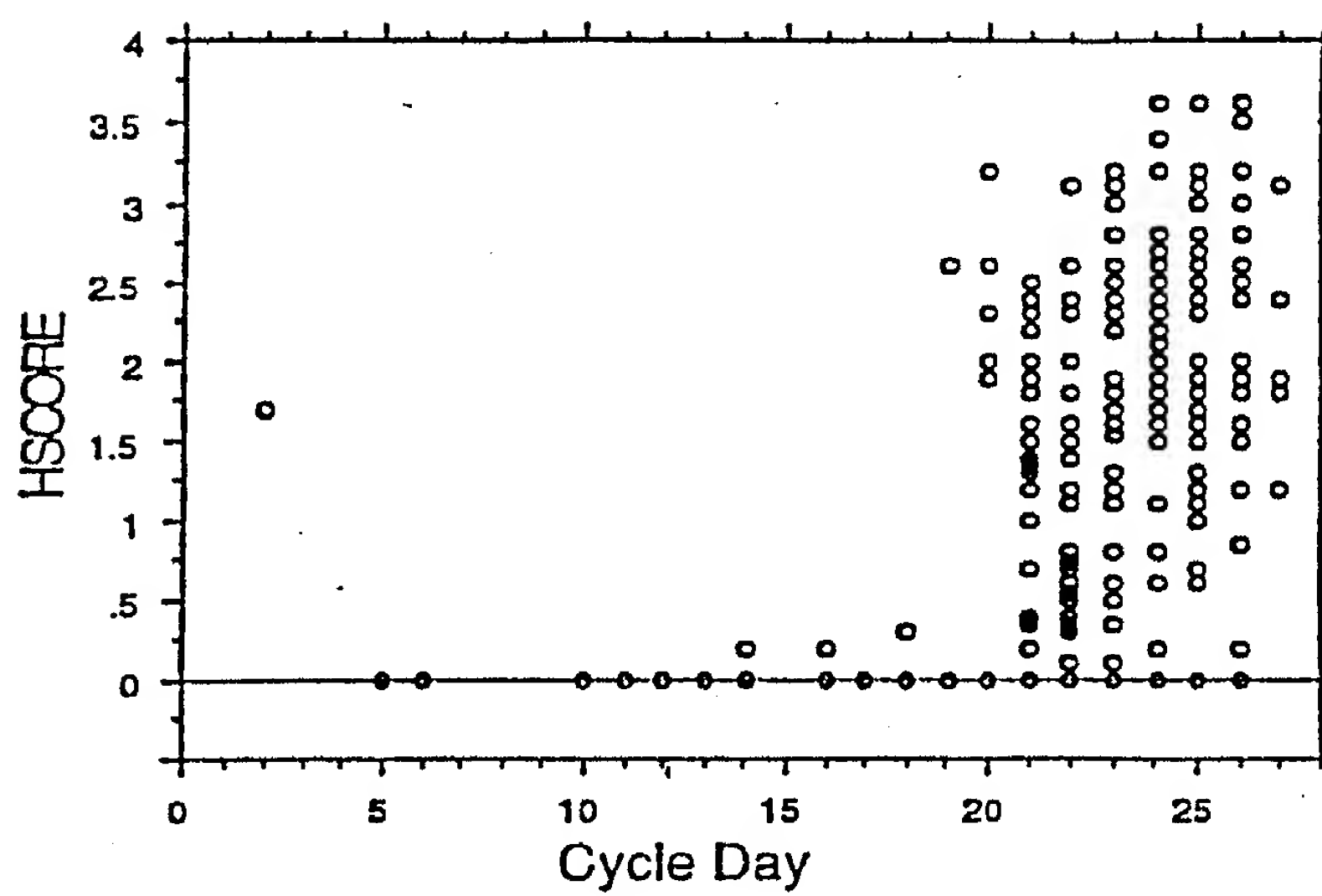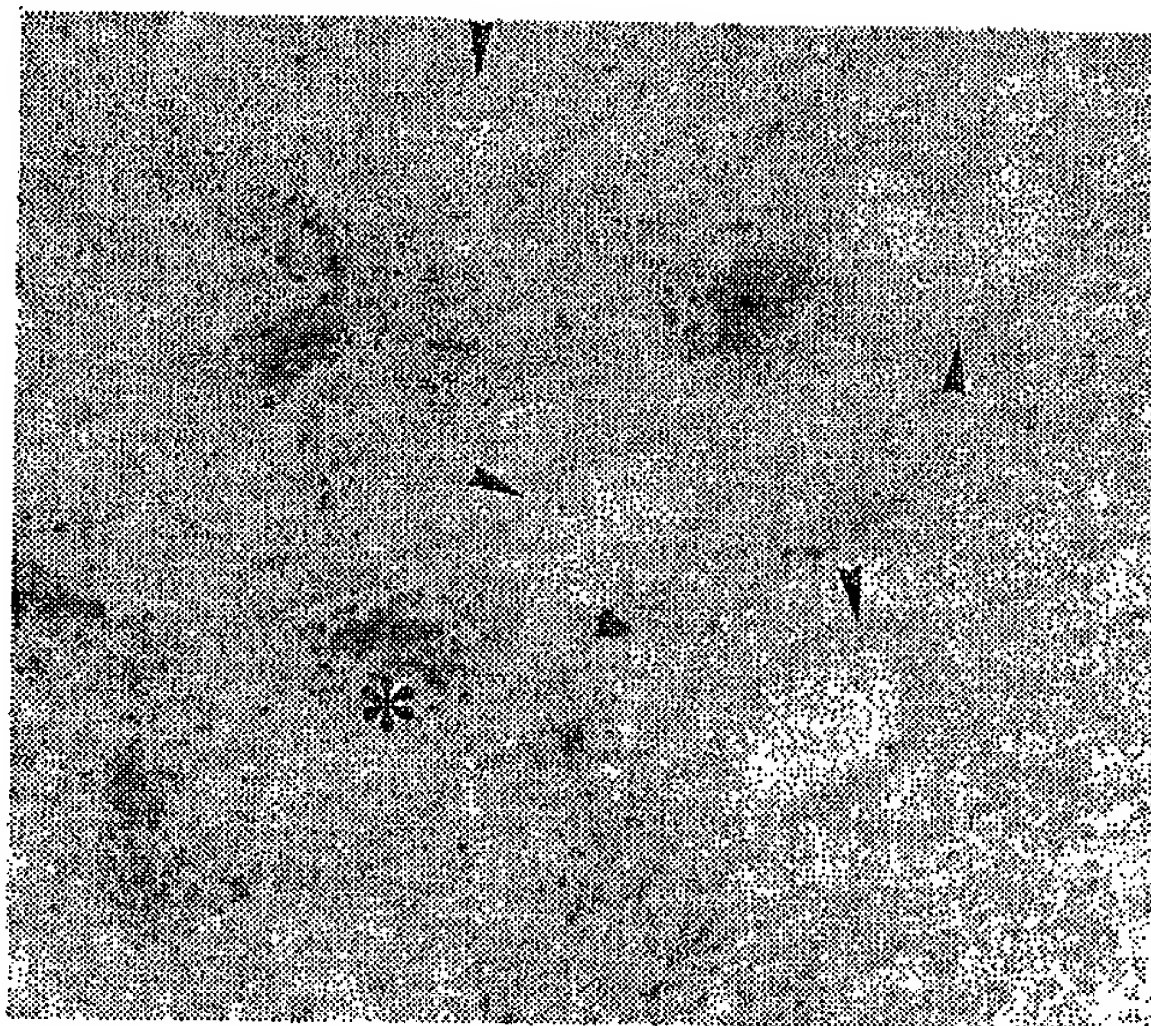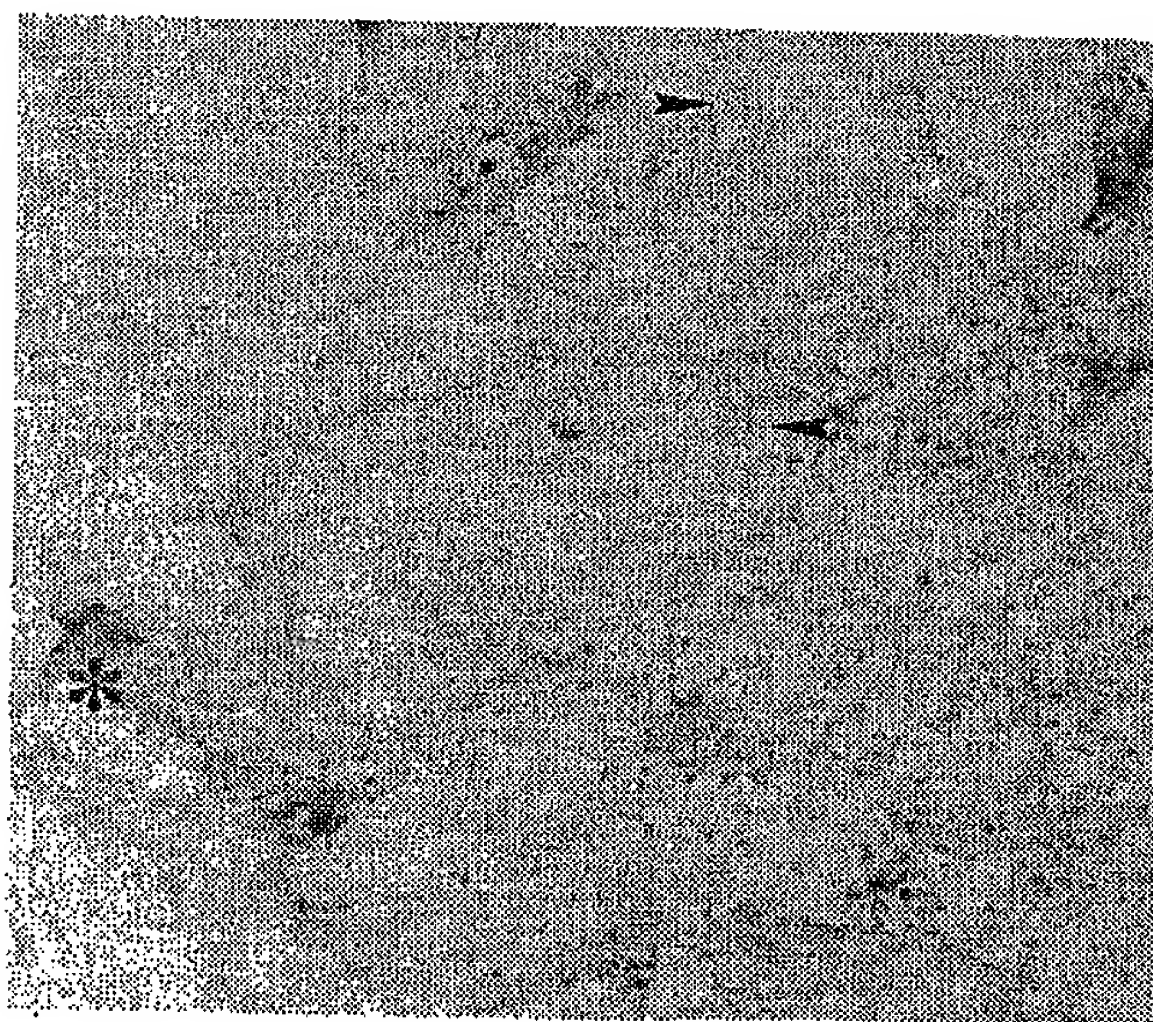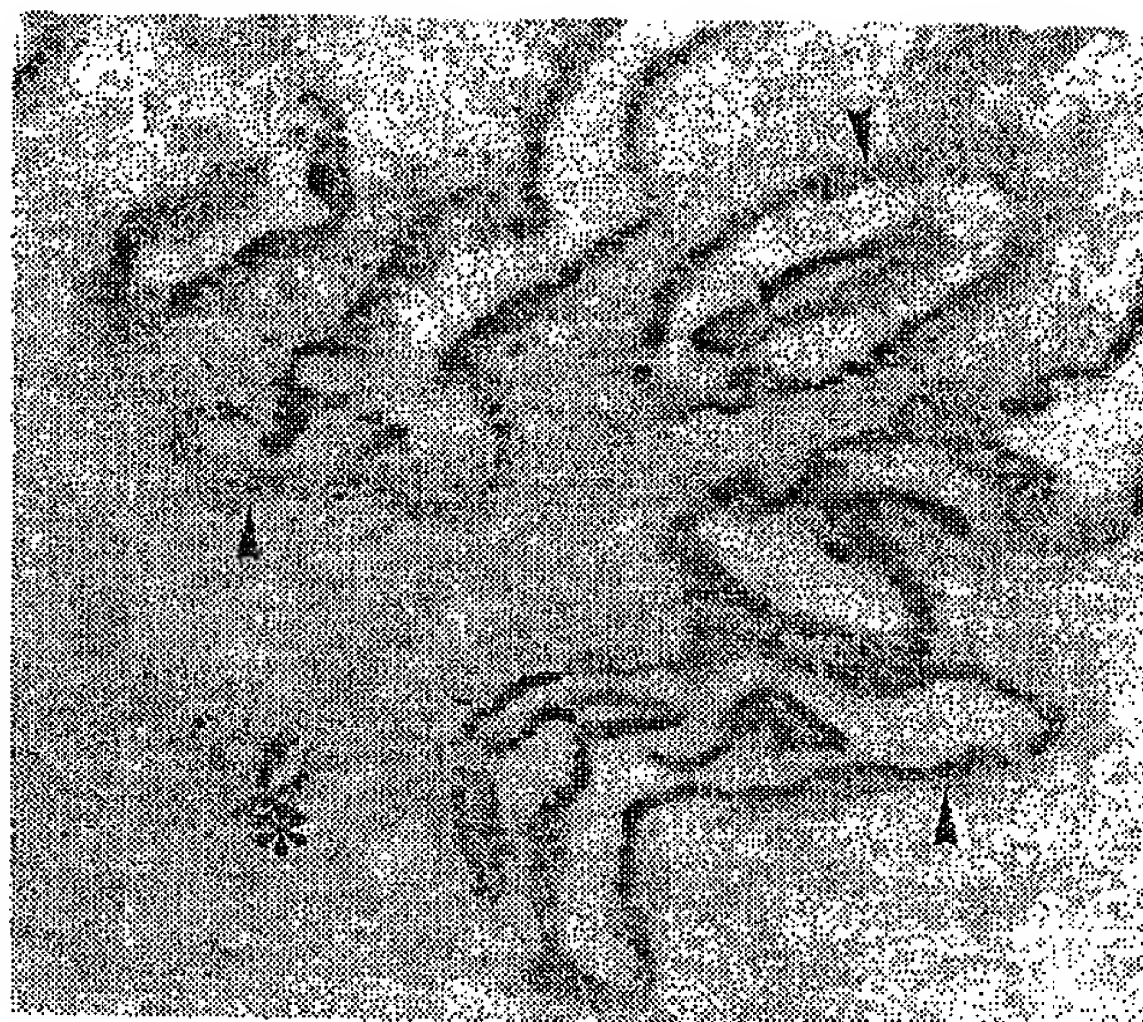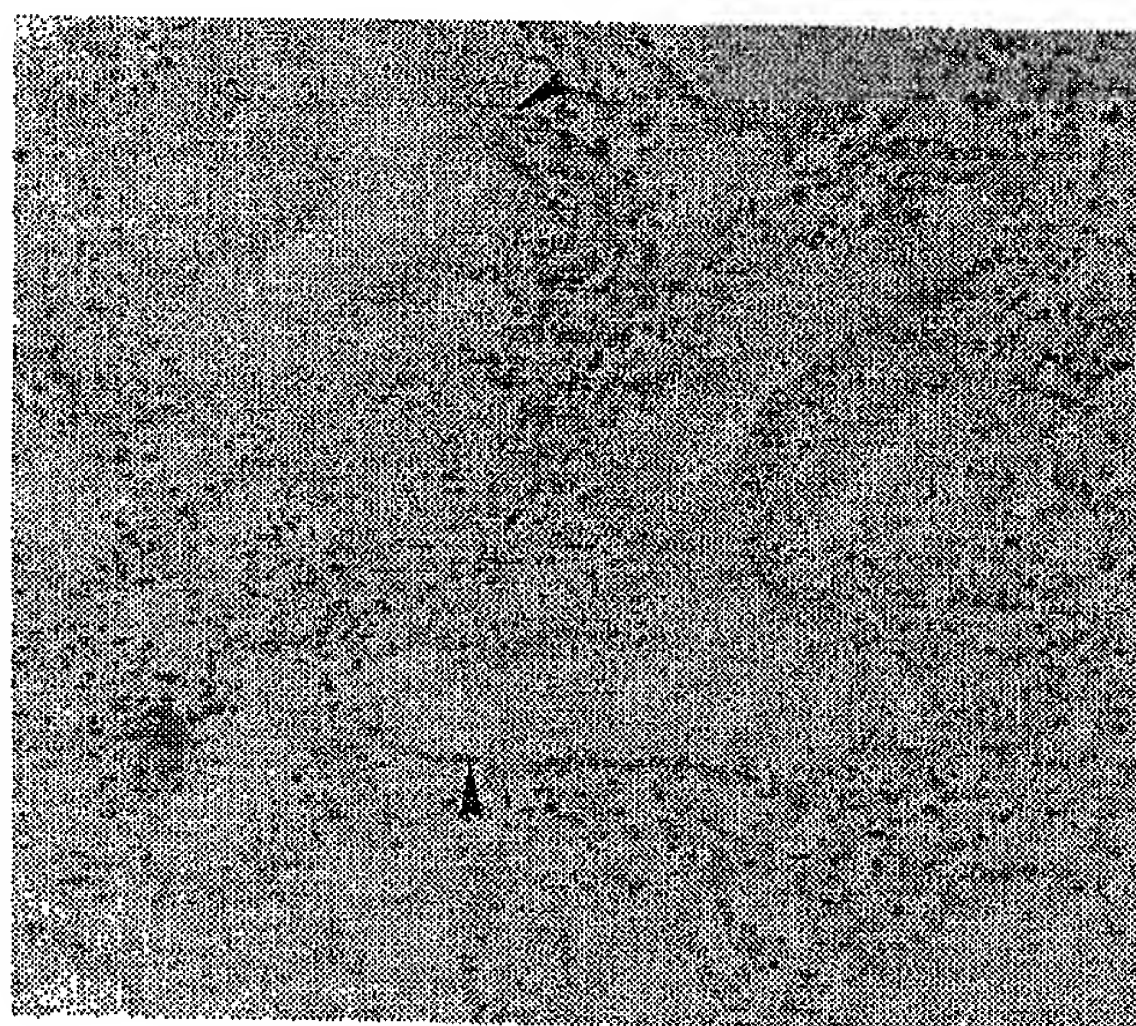
—205

—92

—69

FIG. 6A

1 2 3 4 5

FIG. 6B

FIGURE 7

FIG. 8A

FIG. 8B

FIG.8C

FIG.8D
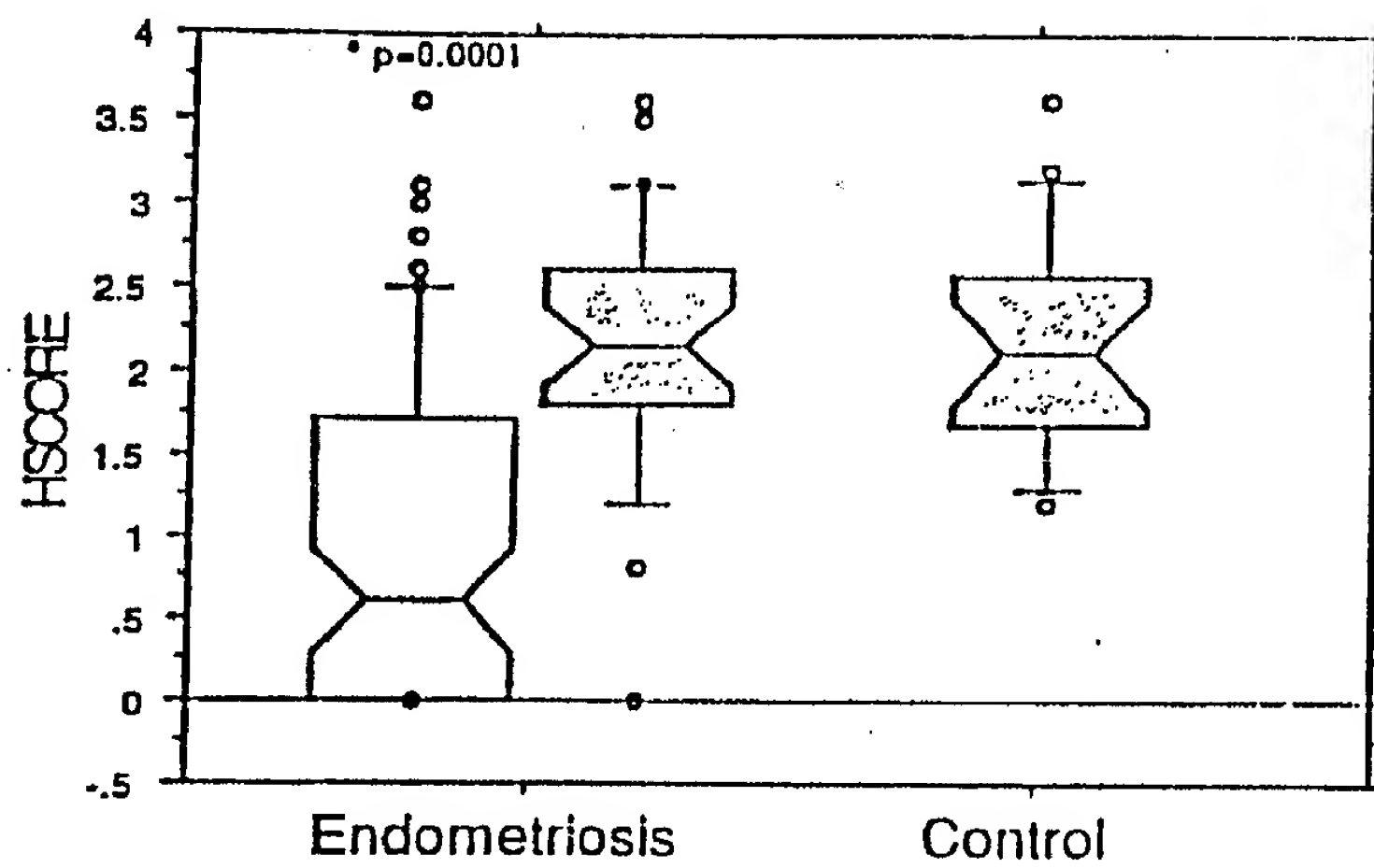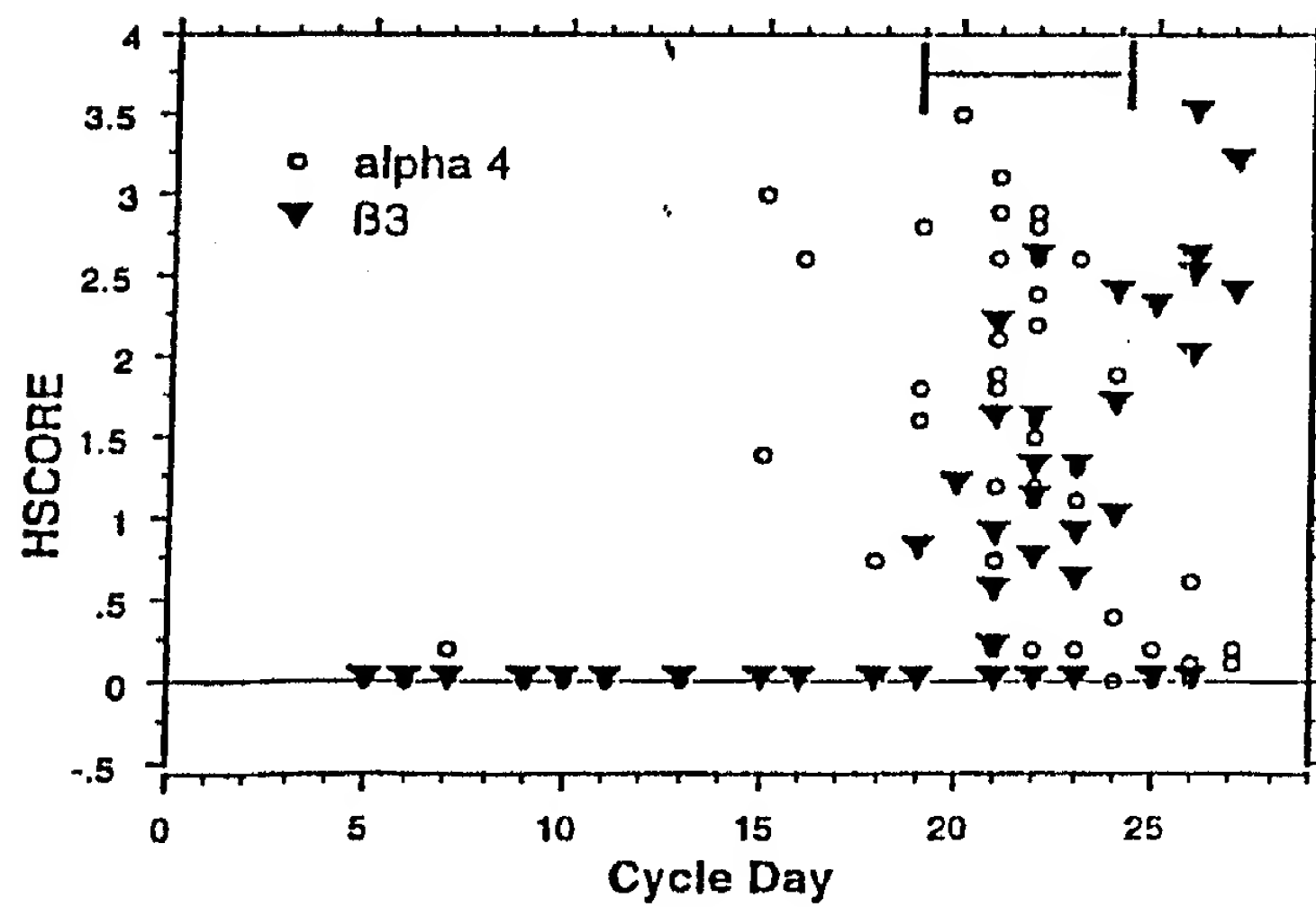
FIGURE 9

FIGURE 10

In....national application No.

PCT/US94/13299

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 35/48, 39/395; G01N 33/15, 33/543, 33/554, 33/577
US CL :424/142.1, 537, 559; 435/7.21; 436/518, 519, 548; 530/388.25
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/142.1, 537, 559; 435/7.21; 436/518, 519, 548; 530/388.25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE
search terms: endometriosis, integrin, vitronectin receptor, monoclonal antibody, hydrosalphinx, contraceptive

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF CLINICAL INVESTIGATION, Volume 90, issued July 1992, LESSEY, B.A., et al, "Integrin Adhesion Molecules in the Human Endometrium Correlation with the Normal and Abnormal Menstrual Cycle", pages 1-8, see entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 FEBRUARY 1995	07 MAR 1995

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13299

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I.   Claims 1-17, drawn to a method of predicting endometriosis using a monoclonal antibody specific for beta 3 integrin.

II.   Claims 18 and 19, drawn to a method of treating a mammal suspected of having endometriosis by inactivating the hydrosalphinx fluid.

III.   Claims 20 and 21, drawn to a method of preventing embryo implantation and a contraceptive composition of hydrosalphinx fluid.

   The claims of Groups I, II and III are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept because the method of Group I requires an antibody which is not required by the methods of Groups II or III, and the methods of Groups II and III require distinct reagents and method steps and have different outcomes.